University of Alberta

The effects of anti-nutritional factors in feedstuffs on gastrointestinal mucin production and on threonine requirement in the growing pig

by

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Abstract

FEED INGREDIENTS THAT STIMULATE MUCIN SECRETION WILL DECREASE THREONINE AVAILABILITY AND INCREASE THREONINE REQUIREMENT IN PIGS

Feedstuffs contain many anti-nutritive factors (ANF), which has the ability to reduce nutrient digestibility and to stimulate intestinal mucus secretion. Mucin protein, a mucus component, is rich in threonine, which is not reabsorbed for utilisation by the animal. Thus, threonine is lost in higher proportions (29-33%) than other amino acids from the small intestine. However, differences in endogenous threonine losses from feeding common feedstuffs are not accounted for in recommendations for threonine intake. Consequently, feeding pigs a typical diet (high in ANF) with threonine just at the requirement level will result in poorer performance due to higher than predicted losses of threonine. Because threonine is the second limiting amino acid in most feedstuffs, including barley, wheat, canola meal, and peas, ANF may increase net threonine loss thereby increasing its requirement. Thus, the objectives of these experiments were:

1) To establish which ANF in common feedstuffs lead to the greatest losses of mucin from the small intestine, hence to the greatest loss of threonine.
2) To demonstrate that the threonine requirement is higher in pigs fed diets with high concentrations of mucin-stimulating anti-nutritive factors.

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Chapter 1
INTRODUCTION

Threonine is an indispensable amino acid that is found in high concentrations in numerous gastrointestinal secretions. These secretory products act to protect gut mucosa from digestive proteases, to prevent dehydration of the underlying mucus membranes and to protect the gut wall from invasion by microbes and parasites (Toribara et al. 1993). Hence, threonine is believed to play a critical role in the development and proper function of the gut. Stoll et al. (1998) have suggested that in piglets 60% of dietary threonine is utilized by the gut, compared to lysine (35%), leucine (32%), and phenylalanine (35%). Additionally, they showed that nearly 90% of this metabolized threonine was either secreted as mucosal protein or catabolized. Further evidence to support this was observed by Chen (1997) and Law (2000). They showed that the threonine requirement for intravenously-fed piglets was 60% lower than that for intragastrically-fed piglets. Because gut atrophy is known to occur as a consequence of parenteral feeding, the dietary requirement for threonine would also be reduced. As threonine is highly involved in the structure and function of gastrointestinal mucus, specifically mucin protein, the difference in requirements during different routes of feeding may be explained by threonine's specific function in the gut. During gut atrophy, there is a decrease in the amount of mucus lining the gastrointestinal tract, which may result in a decreased threonine requirement by the gut, and consequently, a decrease in overall whole body threonine requirement. Law (2000) further went on to show that where gastrointestinal mucin is concerned, the availability and/or use of orally-supplied threonine in the production of mucins was preferred over arterial sources. Thus, these data showed that the role of dietary threonine in the mucus lining of the gut is of utmost importance for gut integrity. Unfortunately, the amino acids in mucin are not recycled for use by the animal (Hoskins 1984, Clamp & Grough 1991) and so need to be replaced by dietary sources.

In most cereal-based pig diets, threonine is the second limiting amino acid. In addition, most cereal grains contain many anti-nutritional factors such as lectins, tannins, and fibre components. These anti-nutritional factors (ANF) have the ability to stimulate intestinal mucus secretion and to reduce dietary nutrient digestibility. Consequently, a combination of all of the above factors means that threonine may become the first limiting amino acid for body protein deposition. Therefore, the objectives of these experiments were: (1) to determine if selective ANF in pig feedstuffs decreased amino acid digestibility and increased mucin excretion, thereby increasing

threonine losses from the ileum. (2) To use the indicator amino acid oxidation (IAAO) method to demonstrate whether threonine requirement is higher in pigs fed a diet with high concentrations of mucin-stimulating ANF. Ultimately, an understanding of the effects of ANF on dietary amino acid requirements will improve the accuracy in formulating and supplementing pig diets. Better diet formulation will result in more cost-efficient production, consistent animal performance, and lower nitrogen excretion in pig manure. To maintain the animal's growth, amino acid intakes must be increased when using feeds that stimulate mucin production.

Chapter 2
LITERATURE REVIEW

2.1 The Relationship Between Threonine and Intestinal Mucins

Threonine is an indispensable amino acid that is found in high concentrations in a number of gastrointestinal secretions, specifically in mucin proteins (Lamont 1992, Specian & Oliver 1991). These secretions act to protect the mucosa from digestive proteases, prevent the dehydration of the underlying mucus membranes, and to protect the gut wall from micro organisms and parasitic invasion (Toribara et al. 1993). Thus, threonine is believed to play an important role in the proper development and function of the young pig gut. Stoll et al. (1998) have suggested that in piglets 60% of dietary threonine is utilized by the gut, compared to lysine (35%), leucine (32%), and phenylalanine (35%). Furthermore, they showed that nearly 90% of this metabolized threonine was either secreted as mucosal protein or catabolized. In another study, Bertolo et al. (1998) empirically confirmed that the apparent needs for dietary threonine between oral and intravenous feeding were significantly different. These data seem logical since gut atrophy is known to occur as a consequence of parenteral feeding, thus the dietary requirement of threonine would also be diminished due to decreased mucin synthesis.

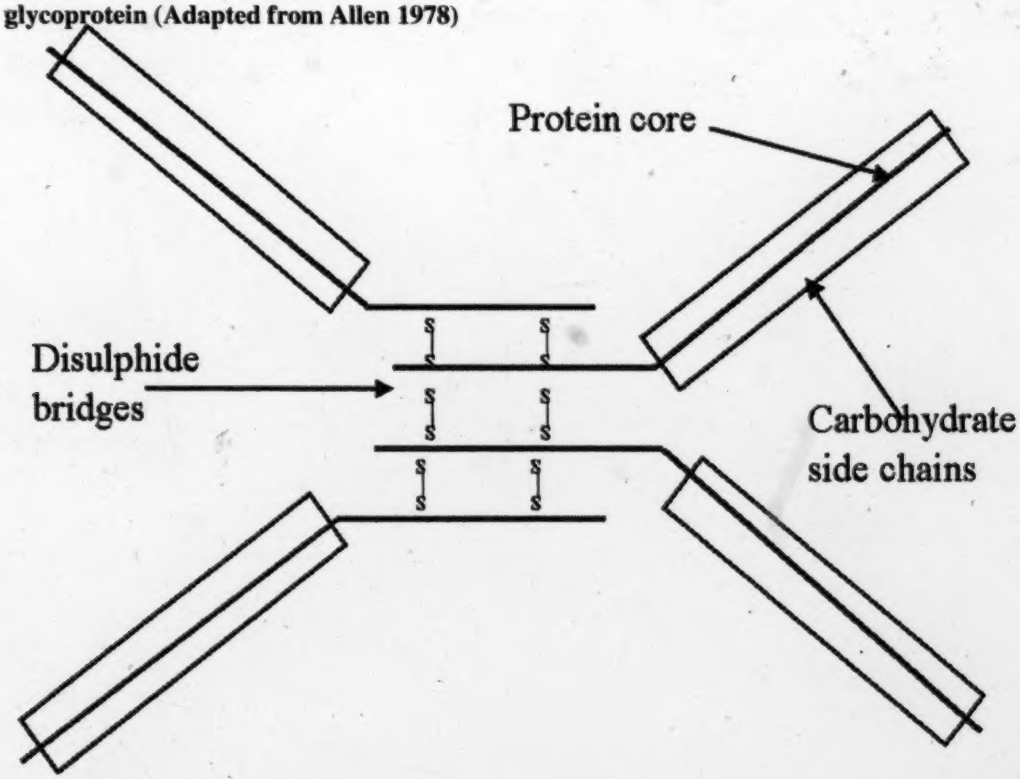
Functions of mucus

Gastrointestinal mucus is exposed to all the physical and chemical forces of digestion. The mucus gels, which cover the entire luminal surface of the gastrointestinal tract, are comprised of water (which represents 95% of the weight of the structure), and materials such as lipids, proteins, ions and enzymes acquired from epithelial secretions and from exfoliated and disrupted cells (Neutra & Forstner 1987). The primary functions of mucus in the gastrointestinal tract are to protect the underlying epithelial cells from (1) corrosive secretions and proteolytic enzymes in the gut, (2) forces generated by digestive processes in the digestive tract, and (3) pathogenic organisms (Neutra & Forstner 1987, Lamont 1992, Toribara et al. 1993). Mucus also aids in digestion by 'anchoring' digestive enzymes thus preventing their rapid removal by peristalsis. That is, mucus creates a digestive zone where digestive enzymes are immobilized near the epithelial surface (Toribara et al. 1993).

Structure of mucus and mucins

The major components of mucus gels are large molecular weight glycoprotein monomers ($2x10^6$ Daltons) known as mucins (Allen 1978) (Figure 2.1.1). Native pig gastric glycoprotein consists on average of four subunits of equal molecular weight ($5x10^5$ Daltons each) joined by disulphide bridges (Snary & Allen 1971). Gastrointestinal mucins are characterized by high carbohydrate to protein ratio with the carbohydrate constituting usually more than 65% of the dry weight (Schrager & Oates 1974, Snary & Allen 1971, Jabbal et al. 1976). The carbohydrate side chains are attached to a protein core. The carbohydrate chains may contain up to five different monosaccharides: galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and sialic acids (Allen 1978, 1981). Pig gastric mucins have longer carbohydrate chains than intestinal mucins with approximately 19 carbohydrate residues per chain compared to 8, respectively (Goodwin & Watkins 1974, Allen 1981, Lien et al. 1997). Consequently, gastric mucins have higher content and different composition of carbohydrates compared to intestinal mucins. However, regardless of the gastrointestinal location of mucin, the carbohydrate side chains are always linked through N-acetylgalactosamine to the hydroxyl groups of either threonine or serine in the protein core. As such, the threonine and serine contents of gastrointestinal mucins are considerably higher than other amino acids (Schrager & Oates 1974, Snary & Allen 1971, Jabbal et al. 1976, Allen 1978). The carbohydrate side chains are closely packed along the protein core; for example, in the pig gastric mucin, on average one in every three or four amino acids carries a chain of 15 sugar residues (Goodwin & Watkins, 1974). Mucin also has high proline content, presumably to straighten the protein core thus allowing close packing of the carbohydrate side chains, resulting in resistance to proteolytic attack (Forstner & Forstner 1986). The protein core is divided into two distinct regions: glycosylated and nonglycosylated regions (Figure 2.1.1). The glycosylated region of the mucin molecule represents over 95% of the glycoprotein, and is known as "native" mucin. Protein in this region accounts for approximately 65% of the total protein of mucin. This protein region is rich in threonine, serine and proline, which make up 40 to 70 mol/100 mol of the native mucin amino acids (Schrager & Oates 1974, Snary & Allen 1971, Jabbal et al. 1976, Lien et al 1997). The nonglycosylated mucin region, termed the "naked" region, represents about 35% by weight of the total protein content or about 4 to 5% of the molecule by weight (Scawen & Allen 1977, Allen 1981).

Figure 2.1.1: Diagrammatic representation of the proposed structure of pig gastric mucus glycoprotein (Adapted from Allen 1978)



The native glycoprotein, that is, with all four subunits has molecular weight of 2.1 x 10^6. The non-glycosylated protein region of the glycoprotein molecule, consist of the disulphide bridges region. The glycosylated protein region consists of the protein core covered by the carbohydrate side chains. The exact arrangement in space of the glycoprotein subunits is not known.

Synthesis and degradation of mucin

The depth of the mucus gel on the mucosal surface is not static; it is the result of a dynamic balance between its secretion and erosion. Allen (1981) proposed that proteolysis, amplified by physical abrasion, is the primary reason for the presence of mucus in the lumen of the digestive tract. Mucin is synthesized in intestinal goblet cells. Goblet cells are more prevalent in the lower than in the upper small intestine, and are derived from the undifferentiated cells lining the intestinal crypts. Evidence suggests that little degradation of mucus occurs prior to the large intestine where it is fermented by enteric bacteria (Hoskins, 1984, Ofosu et al. 1978, Forstner & Forstner 1986, Clamp & Grough 1991). Prior to the large intestine, the glycosylated regions of

mucin protein core are resistant to proteolytic digestion, unlike the naked region of mucin. The proteolytically-resistant glycosylated regions of mucin are dominated by threonine, serine and proline. Overall recovery of mucin at the ileum is greater than 95% (Mantle et al. 1981, Lee et al. 1987). At least 50% of the carbohydrates must be removed before any degradation of native protein occurs (Hoskins 1981, Variyam & Hoskins 1983). Proteolysis of gastrointestinal mucin is associated with a general loss from the pig, of 20 to 30% of the amino acids in mucin, higher for threonine, serine and proline, and little (< 1%) or no loss of carbohydrate (Lien et al 1997, Allen 1981).

Degradation of mucin in the large intestine occurs primarily by bacteria. Unfortunately, amino acids derived from bacterial degradation of mucins are not readily available to the host. Schmitz et al. (1991) injected homoarginine into pigs' caecum and showed that the absorption of amino acids from the large intestine contributed to less than 3% of the total amino acid requirement of the pig. Additionally, several researchers have observed that the amount of nitrogen absorbed in the hindgut for protein synthesis in the pig is low to negligible. The common explanation for this is that protein and amino acids entering the hindgut are converted to ammonia or amines which are absorbed but cannot be used for protein synthesis (Just et al. 1981 Mason et al. 1976, Sauer 1977). Even more recently, van der Schoor et al. (2002), using isotope techniques where they supplied different isotopic threonine tracers intravenously and intraduodenally, were not able to detect any significant recycling of threonine from gastrointestinal mucins, despite the fact that 67% of the total daily threonine intake was utilized by the gastrointestinal tract. Thus, it is apparent that threonine that is used for mucin synthesis is not reabsorbed and so needs to be replaced by dietary threonine. An additional concern is that dietary components may increase mucus secretion, thus an animal's requirement for dietary threonine may be higher when fed diets that increase mucus secretion.

2.2 Effects of Diet on Mucin Secretion

Few studies have examined the effect of individual dietary components on mucin secretion. However, since mucin secretion is stimulated by many of the same neural and hormonal factors that control digestive processes (Neura & Forstner 1987, Mantle & Allen 1989), the diet composition might be expected to have significant effects on mucin secretion. Several researchers have shown that consumption of food increases mucus synthesis. For example, Ohara et al. (1984) showed that the synthesis of gastric glycoprotein is reduced in fasted rats. Kowalewski et al. (1976) reported a 50% increase in the glycoprotein carbohydrate output in Heidenhain pouches

following the consumption of a meal. Sherman et al. (1985) saw substantial reduction in immunoreactive mucin when the food intake in rats was reduced to half their normal daily consumption. Studies by Satchithanandam et al. (1990), Vahouny & Cassidy (1986) and Lien et al. (2001) have suggested that the abrasive action of digestive content - that is, soluble versus insoluble fibre - has an important influence on the amount of mucin entering the large intestine. These researches have also shown that the consumption of different diets will have an influence on the secretion of mucin in both the stomach and small intestine, indirectly via their influence on the digestive processes and directly through their interaction with the mucus gel. In most of these studies, mucus quantity was analyzed by histological, enzymatic, immunoassay or radioisotope techniques. However, because the depth of the mucus layer is the result of a balance between erosion and synthesis, measuring the mucin in this layer may not provide a full account of the effect of dietary treatment on the gastrointestinal mucosa. This misrepresentation is particularly true since animals typically undergo a 24-h fast prior to such measurements (Vahouny et al. 1985, Satchithanandam et al. 1989, 1990). Thus a measure of mucin output from the small intestine may be more suggestive of the effect of dietary treatments on intestinal mucus secretion: although this in itself is not completely indicative of mucin turnover (Mantle & Allen 1989). Estimating the recovery of mucin in ileal digesta would also be beneficial since the presence of this glycoprotein in ileal digesta has often been implicated in the low ileal digestibilities of some amino acids, particularly threonine, observed with many feedstuffs (Sauer & Ozimek, 1986).

Since threonine is an important constituent of the mucus gel lining the epithelial surface, then dietary changes in the intake of this essential amino acid would also likely affect its incorporation into gut mucins. Such a change in threonine availability would ultimately affect the gut's ability to form mucus gel, resulting in alterations in the way the body is protected against ingested pathogens. Within the swine industry, the presence of anti-nutritional factors (ANF) in feedstuffs is a major concern since these ANFs have been shown to increase mucus secretion, subsequently affecting the animals' nutrient intake and availability. One of the more direct concerns is that higher mucus secretion caused by ANF in pig feedstuffs will mean greater threonine losses from the animal. As a result of these losses, a higher threonine intake will be needed by the animal to maintain gut integrity and overall growth and performance.

2.3 Anti-nutritional Factors in Pigs Diets

Pigs require balanced diets that provide adequate levels of all nutrients. Plant-based feedstuffs such as legumes (e.g. soybean, beans), cereal grains (e.g. wheat, barley, corn), and oil seed meal

(e.g. canola meal) are commonly used in pig diets. The nutritional values of these feedstuffs depend, in addition to the chemical composition, on the extent to which nutrients are digested, absorbed and utilized. Anti-nutritional factors (ANF) are substances that cause negative effects on growth, feed conversion efficiency and /or health of an animal, via their ability to hamper digestion, absorption and / or utilization of nutrients. More specifically, ANF may be defined as 'those generated in natural feedstuffs by the normal metabolism of the species from which the material originates and by different mechanisms (e.g. decomposition or activation of some nutrients, diminution of the digestive or metabolic utilization of the food) that exert effects contrary to optimum nutrition' (Chubb 1982).

The list of ANF in plants is quite extensive. They are primarily classified based on the type of nutrients they affect, either directly or indirectly, and the biological response produced in the animal. On this basis they can be divided into four major groups (Chubb 1982, Huisman & Tolman 1992): (1) Substances depressing digestion or metabolic utilization of proteins. This group includes protease inhibitors, lectins, saponins, and polyphenolic compounds. (2) Substances reducing the solubility or interfering with the utilization of mineral elements, e.g. phytic acid. (3) Substances inactivating or increasing the requirements for certain vitamins, e.g. nicotinic acid. (4) Substances that stimulate the immune system e.g. antigenic proteins. However, this review will only focus on some of the major ANF that have been shown to directly affect gastrointestinal mucus secretion, specifically lectins, tannins, and fibre components.

2.3.1 Lectins

Lectins (phytohaemagglutinins) are proteins that are mostly present in the form of glycoproteins, and have the ability to bind to specific sugars or other glycoproteins. The primary effect of lectins is related to their ability to bind to the mucosa of the intestinal wall. This binding can result in damage to the intestinal epithelial cell, resulting in decreased absorption of nutrients, a change in the activity of brush border enzymes and hypersecretion of endogenous protein due to shedding of damaged cells, increased production of mucins and loss of plasma proteins to the intestinal lumen (reviewed by Jaffe 1980, Pusztai 1988, 1991). The biological effects induced by feeding of lectins from kidney beanss and other legumes have been the subject of a considerable number of studies and include an impairment in transport of nutrients across the intestinal wall, intestinal hypertrophy accompanied by an increased rate of synthesis of mucosal protein, and other physiological effects.

Not all lectins produce toxic effects in animals. Within legumes, the toxicity of lectins depends on the species of legumes and also on the animal species. Relative to other beans and legumes, the lectins in kidney beans has been shown to be the most potent anti-nutritional factors in these beans. That is, one can use raw kidney beans (RKB) and state that the toxic effect is caused by the lectins content and not by other ANF that may be present in the bean (Pusztai et al. 1979, 1982, Jaffe 1968, Bond & Smith 1989). These researchers stated that the trypsin inhibitors could not explain the toxic effect of kidney beans because enzymatically digested casein, when added to the toxic diet, did not improve the performance of the experimental animals. Additionally, cyanogenic glycosides were not found to be in toxic amounts in the beans. Hanovar et al. (1962) showed that a kidney beans protein preparation from which the lectins had been removed by affinity chromatography was non-toxic. Pusztai and colleagues (1975) confirmed the finding of Hanovar et al. (1962) when he showed that the net protein utilization (NPU) in lectins-free globulin samples was the same as control (5% casein only), and NPU values were directly related to the level of lectins present. Thus raw kidney beans preparations can be used as a concentrated lectins source to study the anti-nutritional effects of lectins.

The binding of lectins to the mucosa interferes with the absorptive capacity of the intestine. This binding interferes with ion transport as well as the absorption of sugars, amino acids, lipids, and vitamin B_{12}. Lectins also inhibits the activity of certain intestinal enzymes, which participate in the digestion of proteins. Moreover, lectins (from kidney beans) has been shown to cause a rapid increase in the turnover and shedding of the epithelial cells lining the small intestine. A considerable increase in production and secretion of goblet cell mucus has been observed upon feeding rats with kidney beans lectins (King et al. 1980, Pusztai et al. 1982). This effect appeared to be a hypertrophic response since goblet cells numbers were not significantly increased (Pusztai 1988). A high output of mucus would be particularly detrimental to the animal since mucus cannot, to any extent, be reutilized (Hoskins 1984) and therefore would constitute a major net loss of nitrogen, amino acids (especially threonine), and carbohydrate to the animal. Consequently, feeding pigs diets that are high in lectins, such as seen in certain legumes, will lead to significant nutritional concern for the animals' growth and performance.

2.3.2 Tannins

Tannins are polyphenolic compounds, able to form complexes with proteins, carbohydrates and other polymers. Tannins form complexes more easily with proteins than with carbohydrates. This effect is related to the strong hydrogen-bond affinity to the carboxyl oxygen of the peptide group

of proteins. Generally, tannins have the capability to interfere with different aspects of digestive processes via the formation of complexes with digestive enzymes, toxicity after absorption, and damage to the intestinal mucosa (Mitjavila et al. 1977, Jadhav et al. 1989). The effects of tannins can be classified into six main categories: depression of feed intake, formation of tannins complexes with dietary protein and other food components, inhibition of digestive enzymes, increased excretion of endogenous protein (specifically mucus), effect of tannins on the digestive tract, and toxicity of absorbed tannins or their metabolites (reviewed by Jansman 1993, Bravo 1998, Chung et al. 1998).

There are two types of tannins found in nature, hydrolysable and condensed. Hydrolysable tannins have a central core whose hydroxyl groups are esterified to phenolic carboxylic acid. Hydrolysable tannins are readily hydrolysed by acids, alkali or certain enzymes to yield polyhydric alcohol and phenylcarboxylic acid. However, condensed tannins are structurally more complex than hydrolysable tannins: the difficulties in analyzing highly polymerized molecules, the full chemical nature of their structures has yet to be elucidated (reviewed by Jansman 1993, Bravo 1998, Chung et al. 1998). In nature, condensed tannins are more prevalent than hydrolysable tannins. Condensed tannins have been found to be present in a large number (over 500 species) of agriculturally important crops, which are commonly consumed by both animals and humans (Bate-Smith & Lerner 1954, Jadhav et al. 1989, Chung et al. 1998). Tannins are found in a range of plant species, from the major grain crops to the leguminous and sub-tropical forages. For examples, sorghum, millet, barley, rapeseed, and a number of legume seeds (e.g. faba bean) have considerable amounts of condensed tannins and these plants are used extensively for both human food and animal feed. However, not all varieties of these species contain tannins: typical examples are faba bean (Rowland & Corner 1962) and peas (Griffiths 1981), where in both species, condensed tannins content is restricted to those varieties having pigmented flowers. Nevertheless, for monogastric animals, condensed tannins represent a potentially toxic dietary component, which can seriously reduce the digestibility of nitrogen and perhaps other nutritionally essential nutrients (Griffiths 1989).

There have been numerous studies on the effects of tannins in feedstuffs on animal performance (e.g., Cousins et al. 1981, Sell et al. 1985, Mitjavila et al. 1977, Jansman et al. 1993a & b, Yu et al. 1996, King et al. 2000). Some of these studies have been carried out with various feedstuffs, whereas others employed isolated tannins from feedstuffs or standardised commercial tannins, such as tannic acids (available as both hydrolysable and condensed tannins), which were thought

to be representative of tannins in a number of feedstuffs (Jansman 1993). For example, Joslyn and Glick (1968) reported that the feeding of tannic acid to rats at 5% of the diet resulted in lower weight gains (approximately 50% of control). Lease and Mitchell (1940) found that rats could tolerate up to 5% tannic acid mixed in a diet, while higher levels caused a marked growth depression. Experiments with chicks have shown that 0.5% tannic acid in the diet results in growth depression, and high mortality occurs at the 4% level in the diet with sloughing of the mucosa of the oesophagus as revealed by autopsy (Vohra et al. 1966). Mitjavila et al. (1977) showed that rats given 10 g tannic acid (1% of diet) caused hypersecretion of gastric and duodenal mucosa. Wiseman & Cole (1988) conducted a series of studies on the effects of tannins in both pigs and poultry and concluded that tannins level per se had an effect on both apparent metabolizable energy level and nitrogen digestibility, and the tannins level required to elicit a negative effect needed to exceed 1.03 g tannins/d. One of the general conclusions from these studies is that animals can tolerate a certain level of tannins intake before negative effects are observed.

Of course, the results from experiments using whole feedstuffs were more variable. But like the studies above, these results were also related to the tannins levels in the experimental diets. Lizardo et al. (1995) observed that inclusion of sorghum (tannins levels of 1.24 - 1.32% catechin equivalent) caused reduction in protein digestibility and had a negative effect on food intake in pigs. (Note: Tannins are polyphenolic compounds. Catechin (2-flavone-3-ols) is one of the phenols that is found in all tannins; as such catechin equivalent is often used to define the tannic acid content of feedstuffs.). Sell et al. (1985) studied the effects of feeding high - (15% catechin equivalents) and low (5.5% catechin equivalents) -tannins sorghum on the morphology of the duodenum, ileum, caecum and colon of rats and chicks. They found that all intestinal sections were morphologically normal when examined by light microscopy. The only consistent effect appeared to be a slight reduction of the crypt depth and wall thickness of the duodenal tissue in animals fed high tannins sorghum. Jansman et al. (1993) fed high- and low-tannins faba beans to growing pigs, and concluded that 0.6% catechin equivalents of condensed tannins in faba bean (20% faba bean in the diet) reduced the apparent protein and amino acid digestibilities in pigs. Some studies conducted by Liebert & Gebhardt (1983) and Bourdon & Perez (1984) showed that feeding pigs diets that contained 30% faba bean with varying tannins levels (1.0% -1.7%) resulted in negative average daily gain and negative N digestibility, but only at the higher tannins levels. The differences observed in these studies may be due to the methods used for tannins analysis. However, the common observation is that condensed tannins in feedstuffs has negative effects on

animal performance, although more studies need to be done to determine tannins' effect on mucus secretion in pig.

2.3.3 Dietary Fibre

As stated previously, plant products constitute a large proportion of pig diets. In addition to concerns related to the lectins and tannins contents, the level and types of fibre in the animal diets are also a major concern. Dietary fibre is a heterogeneous mixture of structural (cellulose, hemi cellulose, and pectin) and non-structural (gums, mucilages, and algal types) polysaccharides and lignin (Low 1985, Schulze et al. 1994). Shah et al (1982) observed that the addition of fibre to the diet may result in an increase in endogenous nitrogen loss, which they concluded might be due to a number of factors: (1) increased secretion of digestive enzymes; (2) increased sloughing of intestinal mucosal cells; and (3) lowering of intestinal re-absorption of endogenous amino acids secreted in the gut. Also, fibre stimulates proliferation of the mucosal epithelial cells, thus increasing mucosal secretion. Different sources of fibre affect different types of mucosal cells.

High fibre diets have been shown to induce structural, morphological (Sharma & Schumacher 1995, Chiou et al. 1994, Vahouny et al. 1985, Vahouny & Cassidy 1986) and cytokinetic (Jacobs 1986, Satchithanandam et al. 1990) changes in the digestive tract. These changes are consistent with the capability for higher mucin secretion, i.e. more mucin-secreting cells as a result of increased surface area and a larger intestinal tract. Using radioisotope techniques, Vahouny et al. (1985) observed increased mucin secretion in rats fed diets containing cellulose or wheat bran compared to those fed a fibre-free diet. The consumption of citrus fibre, but not guar gum increased mucin secretion in the stomach of rats (Satchithanandam et al. 1989, 1990). Jacobs (1986) showed that the intestine responded to soluble fibre (guar gum and pectin) intake by increasing crypt cell production. These studies suggest that the abrasive action of digestive contents, i.e. insoluble versus soluble fibres, may have an important influence on the amount of mucin entering the large intestine. Lien et al. (2001) also showed that insoluble fibre had a more abrasive action, scraping mucin from mucosa as it passed through the digestive tract. More et al. (1987) also showed that dietary fibre affected mucin secretion. Pigs (60 kg) fed a fibre-free milk-based diet exhibited decreased staining intensities for intestinal mucins compared to a standard diet containing 14.7% fibre (cellulose, hemi cellulose, and lignin). The difference was eliminated when bran was added to the milk diet, indicating that dietary fibre increased the turnover of jejunal mucins. Therefore, fibre does affect mucin production, although this effect depends on the type and quantity of fibre used.

There are various ways of classifying dietary fibre, in addition to the classification of fibre as insoluble and soluble. One popular classification is by the detergent method of analysis developed by van Soest (1963), where fibre is defined as neutral detergent fibre or acid detergent fibre. Neutral detergent fibre (NDF) is a measure of hemi cellulose, cellulose and lignin, representing the fibrous bulk of the forage. Acid detergent fibre (ADF) measures the relative amount of indigestible fractions of cellulose and lignin that are left behind after treating a forage sample with a solution of acid and detergent solution. According to Sauer and Ozimek (1986), the level and source of dietary fibre are the two most important factors influencing the amount of endogenous nitrogen and amino acids present in the ileal digesta. In studies by Sauer et al. (1977) and Taverner et al. (1981), the ileal endogenous protein output increased with the dietary fibre level up to approximately 100 g of NDF/kg of diet but not with further increases. Results by de Lange et al. (1989), Furuya & Kaji (1992) and Schulze et al. (1994) also showed that increases in the levels of dietary fibre did not give additional ileal endogenous flow of N. Lenis et al. (1996) found that the ileal N digestibility decreased by 5.2% with the addition of 15% of purified NDF to the diet of pigs. These data were in good agreement with those of Schulze et al. (1994), who found a decrease of 4.9% in ileal N digestibility with the inclusion of 18% NDF. De Lange et al. (1989), Furuya & Kaji (1992), and Leterme et al. (1992) showed that purified cellulose, as a dietary fibre source had no effect on ileal endogenous N excretion in pigs. So the fibrous constituents, other than cellulose in purified NDF (i.e. hemi cellulose and /or lignin) induced the increased endogenous N secretion.

The emerging picture presented above is that the abrasive actions of insoluble fibre, primarily neutral detergent fibre (even more specifically hemi cellulose and lignin), are mainly responsible for the effect on mucus secretion as measured by ileal digesta and histological techniques. This is an important finding since animal feedstuffs contain all of these fibre components to varying degree. Unlike most of the above studies it is not possible to extract the offensive fibre component from the diet. But, we do need to understand how these fibre components work in normal feedstuffs. As such Table 2.3.1 below shows the fibre components of the three common pig feedstuffs, which will be examined in this project.

In Western Canada, barley, canola meal and wheat (whole grain including the bran) are used quite frequently in swine diets. However, these feedstuffs all have high fibre contents (Table 2.3.1), with fibre components that are known to increase mucus secretion. Although these feedstuffs have similar lignin contents they vary significantly in their levels of ADF, NDF and

hemi cellulose. As such with proper diet formulation these feedstuffs can be used to individually examine the effects of ADF, NDF and hemi cellulose on mucus secretion. Researchers such as Ecknauer et al. (1981) have shown that cellulose has little effect on the rates of mucosal cell division, where as other sources of non-starch polysaccharides (NSP) do. For instance, Southon et al. (1985) found that high NSP diets given to rats induced higher rates of protein synthesis in the jejunum and ileum and more rapid mucosal cell division than in rats given a semi-purified diet containing cellulose as the only source of NSP.

Table 2.3.1: Fibre composition (% of feedstuff, weight as fed basis) of some pig feedstuffs

Component (%)	6-Row barley	Canola meal	Wheat bran
NDF[1 a]	18.6	21.2	42.1
ADF[1 b]	7.0	17.2	13.0
Hemi cellulose	11.6	4.0	29.1

[1]Information obtained from: National Research Council: Nutrient Requirements of Swine (1998). [a] NDF = neutral detergent fibre, which represent ADF and hemi cellulose (NDF-ADF). [b] ADF = acid detergent fibre, which represent cellulose and lignin.

Barley: Barley is said to be an excellent feed for swine, and millions of pigs are raised annually on barley-based diets. In fact, most pigs in Western Canada are raised on barley (Patience et al 1995). Unfortunately, its relatively high crude fibre content (Table 2.3.1) is one of the major reasons for the comparably low energy value associated with barley. Relative to requirements, barley protein is generally low in lysine, isoleucine, threonine, tryptophan and the sulphur-containing amino acids. Barley can be fed as the sole cereal grain (from 25% to 90% of the diet) to pigs from weaning to grower-finishers and to sows (Harrold et al. 1989).

The high fibre content of barley can be grouped as soluble and insoluble fibre. Beta-glucan is one of the main soluble fibres of concern in barley. In chickens, beta glucan can be seen as a major ANF because chickens lack the enzyme to breakdown this molecule. However, although beta-glucan may affect nutrient absorption and digestion in pigs, beta-glucan is not seen as a major ANF in pig diets. Beta glucan represents 3.5 to 4.5% of hulled barley and 4.5 to 7% of hull-less barley (Partridge 2001). Though they have no direct effect on mucus secretion, beta-glucan can slow digesta transition time and cause proliferation of microbes in the foregut of pigs (Partridge

2001). In human nutrition, beta-glucans are viewed as beneficial because they are capable of reducing serum cholesterol and enhance the immune system (e.g. Hallfrisch et al. 1995, Jood & Kalra 2001). However, the bulk of the fibre in barley is insoluble fibre, and as reviewed in the literature above, insoluble fibre has been shown to directly affect mucin output. Phytohaemagglutinins (lectins), protease inhibitors, phytic acids and estrogenic factors are other ANF that are also present in barley: but with the exception of lectins, none of these ANF are directly related to gastrointestinal mucus secretion. However, at the levels at which all of these ANF, including lectins, are presented in barley, their effects are negligible in comparison to the level and effects of insoluble fibre.

Canola Meal: Canola meal is the by-product of oil extraction from canola seeds and contains less than 1.5% oil. Like barley, the greatest limitation of canola meal is its relatively low energy level due to its high fibre content (see Table 2.3.1); canola meal contains about 30% hulls (Thacker 1990). Because canola meal contains up to 40% crude protein, its primary role in pig diets is as a protein source (Thacker 1990). Canola meal is complementary to other feedstuffs used in swine diets and in particular, represents a good "fit" with peas and other pulse crops (Patience et al. 1995). Starting, growing and finishing pigs all benefit from the inclusion of canola meal in their diets at levels of up to approximately 12% of the total diet (Patience et al. 1995). Prior to the general adaptation of the new cultivars of canola, glucosinolates were the major factor limiting the use of canola meal in swine rations (Bell 1984). Canola meal contains the enzyme myrosinase, which is capable of breaking down these glucosinolates into a variety of toxic compounds including isothiocyanates, oxazolidinethiones, nitrile and inorganic thiocyanate ion (Bell 1984). These compounds cause the enlargement of the thyroid gland and inhibit synthesis and secretion of the thyroid hormones (Parik et al. 1980). However, as a result of genetic selection, the level of glucosinolate content of canola meal has been significantly reduced (Bell 1984). In addition, the processing of canola meal inactivates the myrosinase enzyme and prevents the hydrolysis of the glucosinolates, thus rendering them harmless (Bell 1984). Two other groups of compounds in canola meal that may influence its feeding value are tannins and sinapine. Tannins are found at a level of 3% and may have some effect on the digestibility of protein and energy in the diet (Thacker 1990). Canola meal contains about 1.5% sinapine (Mueller et al. 1978), which is a bitter tasting compound that may reduce the palatability of rations containing high levels of canola meal. However, at the levels at which the above mentioned ANF are presented in canola meal, their effects are negligible in comparison to the level and effects of fibre.

Wheat bran: Wheat is grown primarily for human consumption; however it is also used in animals' diets when it is economically feasible due to market conditions or quality discount. Additionally, feed-grade wheat destined specifically for the animal feed market is also grown in various parts of Canada (Patience et al. 1995). But more often, wheat is processed into flour for human consumption and the generated by-products are used for swine diets. The main by-products are wheat shorts, wheat middlings and wheat bran, with bran accounting for 50% of the total by-product. Wheat bran consists mainly of the outer husk of the wheat kernel. It has a low digestible energy due to its high fibre content (Table 2.3.1). Because of its relatively low energy content, wheat bran is not an ingredient of choice in swine diets. However, it may be fed to sows during farrowing when constipation may be a concern. Wheat bran at 5-15% of the diet can be helpful in the prevention of constipation in sows (Patience et al. 1995). On the other hand, wheat bran is a recommended source of fibre for humans wanting to increase their fibre intake. For humans, some of the benefits of increased dietary fibre are decreased risk of colon cancers and decreased plasma cholesterol levels (Niba & Niba 2003). There have been several studies in humans and murine animals showing that wheat bran caused increases in mucus secretion (Sandberg et al. 1981, Dirks & Freeman 1987, Vahouny et al. 1985, Satchithanandam et al. 1990). In most of these studies wheat bran was included at no more than 10-15%, similar to levels offered in swine diets. As such, the inclusion of wheat bran in swine diets is a major concern because of the significant effects on the gut and nutrient intake.

2.3.4 Lignin

Lignin, the second most abundant fibre component within most plants after cellulose, is a complex insoluble polymer and has highly heterogeneous composition (Campbell & Sederoff 1996). Thus, it could be postulated that some of the effects of dietary fibre on the gastrointestinal tract may be due to the large amount of the lignin component of fibre. Additionally, in pig feed, lignin is used as a binding agent during pelleting (Schoeff 1994). Thus, for these two reasons, we need to consider the effect of lignin on the gastrointestinal tract, specifically mucus secretion.

Various researchers have shown that supplementation of fibre components can change the intestinal mucosal tissue. Sigleo et al. (1984) suggested that different fibre components influenced intestinal nutrient absorption and metabolism differently. Within the gut, segments of the jejunum and ileum are major sites of nutrient absorption, and the length of villi correlate positively with nutrient absorption (Pluske et al. 1996). Sigleo et al. (1984) observed that nutrient concentrations were increased in the distal end of the small intestine where more nutrients were available; this

created an apparent change in the mucosal cells of the intestinal segment. Chiou et al. (1994) also observed that lignin significantly decreased the crypt depth of the duodenum in rabbits. The duodenum crypt depth was 93.1 μm for lignin-treated rabbits, while the crypt depths were 164.3 μm and 138.9 μm for rabbits on cellulose and pectin treatments, respectively. Duodenal villus height and muscle layer were not significantly different among treatments. More importantly, Chiou et al. (1994) noted that lignin significantly shortened jejunal villus height (458.3 μm for lignin compared to 707.8 μm and 663.8 μm for cellulose and pectin treatments, respectively) and created a thinner jejunal muscle layer (80.8 μm for lignin compared to 130.0 μm and 165.4 μm for cellulose and pectin treatments respectively). In the ileum, cellulose, pectin and lignin shortened villus height (469.3 μm, 428.8 μm, 452.7 μm, respectively) compared to alfalfa (651.3 μm). Thus, supplementation of lignin can significantly shorten villi in the distal end of the small intestine, thereby decreasing nutrient absorption in the rabbit. Shah et al. (1982) observed that lignin at levels of 3% and 6% in rat diets caused a decrease in body weight, an increase in excretion of fecal nitrogen, and a significant decrease in net protein retention (NPR). In terms of actual percentage, lignin in foods is lower than that of other fibre components. Most studies that analyze dietary fibre components usually include lignin levels of 3-6% because this is the level that is normally present in most food sources (Chiou et al. 1994, Shah et al. 1982)

It is therefore apparent that ANF, specifically lectins, tannins, and fibre components in feedstuffs may result in significant increases in mucus secretion. This would therefore imply an increase in threonine loss because threonine is highly concentrated in mucin and this threonine is not recycled. Because threonine is an indispensable amino acid in animals, the body does not have the capability to synthesize it or its carbon skeleton and thus it must be supplied by the diet. Also, it has been shown that the gut uses approximately 60% of dietary threonine (Fuller et al. 1989, Stoll et al. 1998, Bertolo et al. 1998). Because only 12% of this extracted threonine was found in non-secreted mucosal protein, the remaining 88% was either secreted into the lumen as protein or catabolized within the gut (Stoll et al. 1998). Although the majority of threonine losses by the gut are presumed to be via mucin secretion (Allen 1981, Mantle & Allen 1981, Lien et al. 1997), it is possible that the gut may also catabolize a significant amount of threonine (Wu 1998).

2.4 Threonine Metabolism

The gut, primarily the small intestine, is not only responsible for digestion and absorption of nutrients; it also plays a critical role in amino acid metabolism. In fact, gastrointestinal tissues have one of the highest fractional protein synthesis rates of any tissue in the body (Waterlow 1979, van der Meulen & Jansman 1997). Gastrointestinal mucosal protein synthesis is one of the primary reasons for the gut's high synthesis rate. The cells of the mucosa are capable of utilizing both dietary and arterial amino acids for protein synthesis. However, in the case of threonine, it has been shown that the gut preferentially uses dietary threonine for mucosal protein synthesis (Stoll et al. 1998, Law 2000, van Schoor et al. 2002). These results would suggest that the gut might be an important site, not only for the utilization of dietary threonine, but also in dietary threonine catabolism. However, for pigs, enzymes involved in threonine catabolism have only been isolated from the liver and pancreas, with the main activity site being in the liver (Le Floc'h et al. 1995, 1997). In pigs, there are two main pathways for the catabolism of L-threonine. Threonine dehydratase (TDH) and threonine dehydrogenase (TDG) are the two major enzymes, which are located in different cellular compartments (mitochondria and cytosol, respectively); both enzymes are capable of assuming a dominant role in the catabolism of threonine during different metabolic states (Bird & Nunn 1983). (Figure 2.4.1)

Threonine Dehydrogenase (see Figure 2.4.1)

The catabolism of threonine using threonine dehydrogenase (TDG) pathway leads to the formation of 2-amino-3-ketobutyrate, which may spontaneously decarboxylate to form amino acetone; but the major fraction (> 90%) seems to be cleaved to glycine and acetyl-CoA (Bird & Nunn 1983). Because preparations of rat liver mitochondria lacking threonine aldolase activity produced glycine from L-threonine, the reaction probably involved the coupled activities of threonine dehydrogenase and 2-amino-3-ketobutyrate CoA-ligase (Bird & Nunn 1983). At low concentrations of L-threonine the rate of glycine production was several times greater than the rate of amino acetone formation. Glycine may be oxidized by the mitochondrial glycine-cleavage system, or incorporated into a number of important cellular constituents (Neuberger 1981). Acetyl-CoA might be oxidized via the tricarboxylate cycle, or transported from mitochondria via citrate for use in lipid synthesis. TDG activity appears to be dominant during the normal-fed state, when the requirement for hepatic gluconeogenesis is low (Bird & Nunn 1983). In this condition, the physiological role of the dehydrogenase activity is probably the homeostasis of free somatic threonine concentrations derived ultimately from dietary threonine (Bird & Nunn 1983). Thus, in

the normal-fed rat, the function of threonine dehyrogenase might be simply to partition excess threonine carbon for oxidative or biosynthetic purposes.

TDG remains the major oxidative pathway in both starved and protein-free fed pigs, but threonine was more efficiently conserved in the protein-free group in which threonine oxidation was reduced to a minimum (12% of total threonine oxidation) (Ballevre et al. 1991, Le Floc'h et al. 1995). In fed pigs, 76% and 80% of threonine oxidation occurred through glycine formation in the low threonine and high threonine treatments, respectively (Ballevre et al. 1991, Le Floc'h et al. 1995). Therefore, TDG is the major oxidative pathway in fed growing pigs.

In humans, compared to rats and pigs, the partition of threonine oxidation between the TDG and TDH pathways is different (Darling et al. 2000). In human infant TDG activity accounted for 44% of total threonine oxidation (Darling et al. 1999), however, in adult humans, TDG activity accounted for only 7-11% of total threonine catabolism (Darling et al. 2000). They speculated that the differences seen in adult human versus infant may be related to a higher metabolic requirement for glycine in infants compared to adults (Darling et al. 2000). Therefore, it would seem that in growing animals there is a greater demand for glycine which is partially satisfied through threonine, consequently a greater need for TDG activity in growing animals and humans.

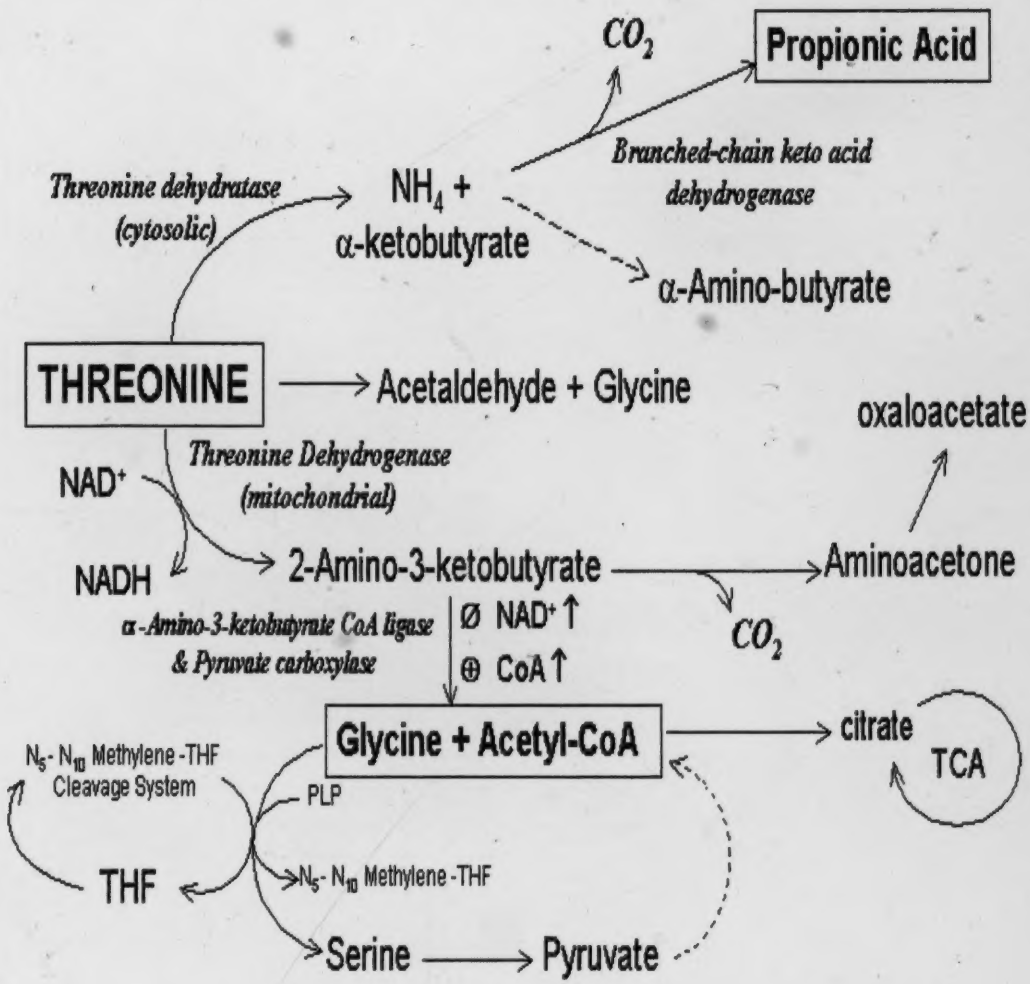
Threonine Dehydratase (see Figure 2.4.1)

Catabolism of threonine using the threonine dehydratase (TDH) pathway leads mainly to propionyl CoA and carbon dioxide, since 2-ketobutyrate is rapidly metabolized by the branched-chain ketoacid dehydrogenase (Bird & Nunn 1983). The propionyl CoA can be converted to succinyl CoA and therefore, via the citric acid cycle, can lead to gluconeogenesis from threonine. The major physiological function of TDH is to mobilize threonine carbon for hepatic gluconeogenesis (as occurs when feeding a high-protein diet or during starvation) (Mak et al. 1981). Threonine dehydratase is inducible with changes in feeding and is believed to be involved in the homeostatic regulation of free threonine concentrations (Ishikawa et al. 1975). While TDH plays a major role in the gluconeogenic pathway of rats it represent only 1-3% of TDG activity in pigs (Le Floc'h et al. 1995).

Although the whole body catabolism of threonine in pigs has been described, the enzymes have been localized only to the liver and pancreas (Ballevre et al. 1991, Le Floc'h et al. 1995, 1997, Bird & Nunn 1983). To the best of the authors' knowledge, the activity of these enzymes has not

conclusively been reported for the intestine; although there had been some tentative work by Simpson et al. (1998), which suggested that there might be some TDH activity in the duodenum of the growing pig. However, previous data on mucosal activity of enzymes have been compromised by inadequate control of endogenous protease activity (Wu 1998). Additionally, TDH activity plays a very minute role in threonine catabolism in pigs (Le Floc'h et al. 1995).

Figure 2.4.1: Threonine metabolism in pigs



For pigs, threonine (THR) is catabolized by threonine dehydatase (TDH) and threonine dehydrogenase (TDG). The primary end products of the THR using TDH are carbon dioxide (CO_2) and propionic acid. The primary end products of THR, using TDG are glycine and acetyl-CoA. Other abbreviations: NAD/NADH – nicotinamide adenine dinucleotide, NH_4 – ammonium, TCA – tricarboxylic acid cycle, THF – tetrahydrofolate, and PLP – pyridoxal phosphate.

2.5 Estimates of Threonine Requirements

Threonine is an indispensable dietary amino acid, which has great nutritional and physiological importance for humans and other animals. Growth, nitrogen balance, plasma amino acid concentrations, and amino acid oxidation are the more often used methods to estimate amino acid requirements. Each of these methods has been subjected to criticisms for various methodological and theoretical reasons (e.g., see reviews by Fuller & Garlick 1994, Pencharz & Ball 2003).

Traditional methods (Weight gain, Nitrogen balance and Plasma concentration):
Growth performance is the simplest criteria used to gauge an animal's response to a dietary treatment. Nutrient deficiency usually results in reduced feed intake and impaired growth. For example, Kang-Lee & Harper (1978) saw that in rats, weight losses due to threonine deficiency were more severe than weight losses due to deficiencies of several of the other indispensable amino acids. However, growth and feed efficiency per se do not provide metabolic information relating to nutrient requirements. That said, weight gain and feed efficiency have been used as the primary assessment criteria for amino acid requirement for pigs (Rosell & Zimmerman 1985, Lewis & Peo 1986, Leibholz 1988). In fact, the National Research Council's (NRC 1998) amino acid requirements for pigs are based predominantly on weight gain and feed efficiency research, along with some work using nitrogen balance.

Nitrogen balance experiments are the classical method for determining amino acid requirements in human and other animals. In the historic works of Rose and colleagues (Rose 1957) they did a series of nitrogen balance studies in adult men to determine the essential amino acid requirements of humans. Similar nitrogen balance studies have also been used in other animals, such as swine, to determine their amino acid requirements in both non-growing and growing states. For example, Brown and Cline (1974) used urea nitrogen excretion as an indicator of lysine and tryptophan requirements in pigs. Recent work by Heger et al. (2002), involved the use of nitrogen balance experiments in growing pigs to estimate the maintenance requirement for several indispensable amino acids, including lysine and threonine. However, nitrogen balance studies tend to overestimate the true rates of body nitrogen retention because of measurement errors in collection of feces and/or urine samples, errors in determining intake, and not accounting for nitrogen losses as ammonia gases or through the skin/hair (Fuller & Garlick 1994). Additionally, nitrogen equilibrium is taken to imply amino acid equilibrium; however, this is not always true. Rather, the amino acid composition of the experimental animal's body may undergo substantial changes to compensate when an amino acid deficient diet is given (e.g., histidine) (Fuller & Garlick 1994).

Another problem with nitrogen balance studies is usually the limited number of observations, due to the time needed for adaptation to each new intake level. Later studies using radioisotope techniques have shown that nitrogen balance results are generalized estimations of amino acid requirements, even in well-controlled nitrogen studies (Zello et al. 1995, Kim et al. 1983a & b).

Measurement of plasma amino acid concentration is another method for studying amino acid requirement. For instance, Kang-Lee & Harper (1978) reported that in rats fed diets with graded levels of threonine, plasma threonine concentration remained low until dietary threonine content exceeded 0.3%, then increased gradually until dietary threonine content reached 0.55%, whereupon plasma threonine concentration increased sharply with each further increment of threonine. In humans, plasma threonine concentrations showed a progressive decline with the decrease in dietary threonine intake in both fed and post-absorptive state (Zhao et al. 1986). Both plasma threonine concentration and threonine oxidation have been used to determine the requirement (Zhao et al. 1986) in young adult human subjects; however, plasma levels of amino acids are the sum of many homeostatic processes controlled primarily by the liver (Ball et al. 1986). Additionally, there are many confounding factors that contribute to changes in plasma amino acid levels including: age, timing of sampling, length of adaptation to the diets, and the specific amino acid under investigation (Scrimshaw 1996). Thus, plasma amino acid concentrations are not considered to be highly sensitive measures for determining amino acid requirements.

Indicator amino acid oxidation (IAAO)

In comparison to nitrogen balance and plasma amino acid concentration techniques, the indicator amino acid oxidation technique is a more sensitive measure of amino acid requirements (House 1997, 1998, Chen 1997, Law 2000). Direct amino acid oxidation has been used to estimate threonine requirement (Kang-Lee & Harper 1978, Zhao et al. 1986). However, because threonine carbons 1 and 2 are sequestered by glycine, threonine oxidation is greatly underestimated when measured by the production of labelled CO_2 (Ballevre et al. 1991). In this regard, the indicator amino acid oxidation technique has proven more accurate to measure threonine requirement (Kim et al. 1983a & b, Chen 1997, Law 2000). Indicator amino acid oxidation is based on the assumption that the level of the limiting amino acid determines the partition of any indispensable amino acid between retention for protein synthesis and oxidation. Consequently, when only one indispensable amino acid is limiting for protein synthesis, the other amino acids will be oxidized because they are in relative excess. As the dietary level of the limiting amino acid (test amino

acid) increases, the incorporation of the other dietary amino acids into protein will also increase, thereby decreasing their oxidation. This process will continue until the requirement level of the test amino acid is reached, at which point further additions of this amino acid will result in its oxidation rather than being stored as protein (Ball et al. 1996, Kim et al. 1983a & b).

The indicator amino acid oxidation method has been used to determine the threonine requirement in orally-fed pigs (Kim et al. 1983a & b). Increasing the dietary threonine level from 4 to 6 g/kg diet resulted in a linear decrease in the release of labelled carbon dioxide from labelled phenylalanine, the indicator amino acid. However, increasing the threonine level to either 6.5 or 8.0 g/kg caused no further reduction in $^{14}CO_2$ released by the pigs. An intake of 6 g threonine/kg diet (~ 0.58 g threonine/ kg body weight/ d) was therefore suggested as the amount required to maximize amino acid retention for 2.5 kg pigs (Kim et al. 1983). Using the same technique Chen (1997) determined that intravenously-fed piglets (8 days old) had a threonine requirement of 0.21 g threonine/kg/ d. Subsequently, also using IAAO technique Law (2000) determined the threonine requirement for intragastrically-fed piglets (8 days old) to be 0.55 g/kg/ d, which was not different from the estimate of Kim et al. (1983). This dramatic difference between orally- and intravenously-fed pigs was probably related to lower mucin synthesis by the substantially diminished intestinal function of the parenterally-fed piglets (Bertolo et al. 1998).

Phenylalanine as a suitable indicator

In most indicator amino acid oxidation studies, [^{14}C]-phenylalanine has been used as the indicator, in which phenylalanine oxidation has been demonstrated to be inversely related to protein synthesis (Ball & Bayley 1984). Phenylalanine is a dietary indispensable amino acid, with its routes of appearance in metabolic pools restricted to dietary intake and endogenous protein breakdown. Routes of disappearance of the carboxyl carbon are protein synthesis and oxidation to CO_2 by way of tyrosine catabolism. Conversion of phenylalanine to tyrosine is the primary step in phenylalanine oxidation pathway. Several researchers (Zello et al. 1995, House et al. 1997, 1998) have shown that the exchange between labelled tyrosine (in the liver mitochondrial from phenylalanine oxidation) and unlabelled tyrosine (in plasma and other tissues) are minimized. Human studies (Zello et al. 1995) in which ^{13}C-phenylalanine was the indicator and excess dietary tyrosine was provided, have shown a minimal transfer of label from phenylalanine to tyrosine (mean of 3.4%) over a wide range of phenylalanine intakes. Similar results were observed in pig studies using ^{14}C-phenylalanine (House et al. 1997). House et al observed that when sufficient dietary tyrosine (54% above phenylalanine requirement) is available then

conversion of labelled phenylalanine to the unlabelled tyrosine pool was minimized. Additional routes of phenylalanine disappearance are quantitatively minor and limited to the production of thyroid hormones, melanin, and catecholamine and therefore unlikely to affect measurements of phenylalanine oxidation.

Phenylalanine is catabolized primarily in the liver, suggesting that the liver could function to regulate peripheral tissue exposure to dietary phenylalanine intake. Liver uptake of phenylalanine was the most responsive of the indispensable amino acids to exogenous protein intake (Neale & Waterlow 1974). Plasma phenylalanine concentration showed the least change of any indispensable amino acids in piglets that were fasted or fed protein-free diets (Shimada & Zimmerman 1973). Additionally, the free phenylalanine pool in the liver is small and closely regulated, implying that the level of oxidation would be affected by amino acids entering the liver after a meal. Also, phenylalanine oxidation is most sensitive to changes in the dietary level of other amino acids when dietary phenylalanine is consumed closest to its requirement (Kim et al. 1983). Combined, these reasons have confirmed the use of phenylalanine as an indicator of test amino acid deficiency. In addition, the IAAO technique has been proven as an appropriate approach to the study of amino acid requirements of piglets.

2.6 Protein and Amino Acid Digestibility in Feedstuffs

The protein level necessary to provide an adequate intake of essential amino acids will depend on the feedstuff. Cereal grains such as barley and wheat are the primary ingredients in most swine diets and usually provide 30 to 60% of the total amino acid requirements, with lysine usually as the first limiting and threonine as the second limiting amino acids (NRC 1998). Also, in most swine feedstuffs, a proportion of each amino acid that is present may not be biologically available to the animal, because the feedstuff may not be fully digested and/or absorbed. As such, it is more desirable to express amino acid requirements in terms of bioavailability. That is, in formulating pig diets, the amino acid bioavailability of the ingredients needs to be considered.

Within feedstuffs, amino acid digestibility does not equate to amino acid availability, and since amino acids are usually the most expensive component of pigs' diets, there have been extensive studies to evaluate amino acid digestibilities for many feedstuffs (Sauer & Ozimek 1986, Sauer et al. 2001). Availability is defined as the proportion of the amino acid in the diet that is absorbed in a form suitable for utilization by the animal (Sauer & Ozimek 1986). The primary method to determine availability has been to measure the proportion of dietary amino acid that disappeared

from the gut when digesta reaches the terminal ileum. Thus, amino acid digestibility has been defined as the difference between the amount of amino acid in the diet and in ileal digesta divided by the amount in the diet (Sauer & Ozimek 1986). Values determined by this method, and without correction for endogenous amino acid losses, are termed apparent ileal digestibilities rather than bioavailabilities. Corrections of ileal digestibility values for estimated endogenous losses are termed 'true' or standardized ileal digestibility (Sauer et al. 2001, Moughan 2003). However, these estimates of endogenous losses are often determined using protein-free diets and do not account for the differences in endogenous losses among feedstuffs. The variable ANF contents of feedstuffs are known to affect mucus secretion and hence endogenous amino acid losses, therefore the estimates used by NRC to correct apparent ileal digestibility are inaccurate and need to be improved or replaced. Amino acid availability values for feedstuffs have also been determined by methods such as growth assay (Kovar et al. 1993, Adeola et al. 1994), and more recently by the indicator amino acid oxidation (IAAO) technique (Ball et al. 2002). With the IAAO method, 'true' amino acid availability for protein synthesis in specific feedstuffs can be calculated directly from phenylalanine oxidation without the need for indirect calculation relating to dietary digestibilities or endogenous losses. Additionally, the IAAO method is more rapid and cheaper than the growth assay (Ball et al. 2002). The IAAO method allows for more accurate formulation of pig diets, hence less nitrogen excretion and better animal performance.

2.7 Summary

The above literature has shown that the gut takes up threonine to a greater extent than other amino acids, suggesting that the gut itself uses a large proportion of the dietary threonine. This review has also shown that mucin plays a major protective role in the gut. In addition, mucin is affected by dietary changes, and its synthesis most likely depends on threonine intake since this indispensable amino acid is critical to the structure and function of mucin proteins. Therefore, accurate determination of threonine requirement for the growing pig is critical in order to achieve optimum performance and development. In swine diets, threonine may become the first limiting amino acid for body protein deposition due to the presence of mucus stimulating anti-nutritional factors in most feedstuffs. Consequently, when formulating pig diets for threonine, the ANF content and the true amino acid availability of feedstuffs need to be considered.

Chapter 3
RATIONALE, HYPOTHESES and OBJECTIVES

3.1 Rationale

In most pig feedstuffs, threonine is the second limiting amino acid (NRC 1998). Additionally, most pig feedstuffs are rich in anti-nutritional factors (ANF) such as lectins, tannins, and fibre components (Liener 1990, Huisman & Tolman 1992). These ANF act to stimulate gastrointestinal mucus secretion (Pusztai 1988, Jansman 1993, Satchithandam et al. 1990, Lien et al. 1997). Mucin, the major glycoprotein component of mucus is rich in threonine (Allen 1981, Lien et al. 1997). This threonine is excreted into the large intestine and is not recycled for use by the animal (Hoskins 1984, van der Schoor et al. 2002). As such, ANF-rich feedstuffs have the potential to increase the threonine requirement of the animal through increased threonine losses. Consequently, threonine in many pig feedstuffs may become the limiting amino acid for protein deposition based on its bioavailability from the feedstuff along with the presence of mucin-stimulating ANF in the feedstuffs. However, current information on feedstuffs does not account for variation between feedstuffs and the effects of ANF on endogenous amino acid losses. Therefore it must be determined whether feedstuffs and ANF affect threonine requirements. The objectives of these experiments were to determine whether ANF in feedstuffs increase mucus secretion and if so, does this increase the dietary threonine requirement when feeding such feedstuffs.

3.2 Hypotheses

1. Common anti-nutritive factors, such as lectins, tannins, and fibre components in feedstuffs increase mucin excretion leading to an increased loss of threonine from the small intestine.
2. Threonine requirement is higher for pigs fed a diet high in ANF in comparison to pigs fed low ANF, casein-based diet.

3.3 Objectives

1. To establish which ANF (lectins, tannins, fibre, and lignin) in common feedstuffs would cause the greatest mucin output from the small intestine, and therefore potentially contribute to the greatest losses of threonine.
2. To determine whether the threonine requirement is higher in pigs fed diets with high concentrations of mucin-stimulating anti-nutritive factors compared to a low ANF diet.

Chapter 4

MUCIN PRODUCTION IN GROWING PIGS FED SEVERAL ANTI-NUTRITIONAL FACTORS AND FEEDSTUFFS COMPARED TO A CASEIN-BASED CONTROL DIET

4.1 Introduction

Mucin, the primary glycoprotein in gastrointestinal mucus, is rich in threonine (Lien et al. 2001, Allen 1981). Threonine, and sometimes serine, is used for the attachment of carbohydrate side-chains to the protein core of mucin. The mucin molecules form the gel structure of mucus, and are essential for the protection of the gastrointestinal tract from digestive enzymes, mechanical abrasion during digestion and pathogens (Toribara et al 1993). The protein core of mucin is excreted to the large intestine and these amino acids are not available for reuse by the animal (van der Schoor et al. 2002). Additionally, threonine, an indispensable amino acid, is the second-limiting amino acid in most swine feedstuffs including barley, canola meal, peas and wheat (NRC 1998). Because mucus-stimulating anti-nutritive factors (ANF) are found in all of these feedstuffs, there is the possibility that the threonine requirement for swine may be increased when feeding ANF-rich feedstuffs. Consequently, feeding pigs ANF-rich feedstuffs may cause threonine to become the first limiting amino acid for swine with respect to availability for protein deposition. Several researchers (Stoll et al. 1998, Bertolo et al. 1998) have shown that in pigs, the threonine requirement was 60% lower during intravenous feeding compared to oral feeding. These researchers speculated that this might be due to lowered mucin production as a result of gut atrophy.

The more common mucin-stimulating ANF in feedstuffs are tannins, lectins and fibre (Jansman 1993, Pusztai 1988, Sauer & Ozimek 1986, Satchithanandam et al. 1990). For this experiment we looked at three purified ANF components (lignin, tannins and lectins) and three common feedstuffs that have medium to high fibre contents (wheat bran, barley and canola meal). All six treatments were included at levels expected to occur in common pig diets.

Thus, we hypothesized that threonine requirement is affected by losses via mucin excretion and hence will be different when different feedstuffs are fed to pigs. To test our hypothesis, the objective of this first experiment was to determine whether or not anti-nutritive factors (lectins,

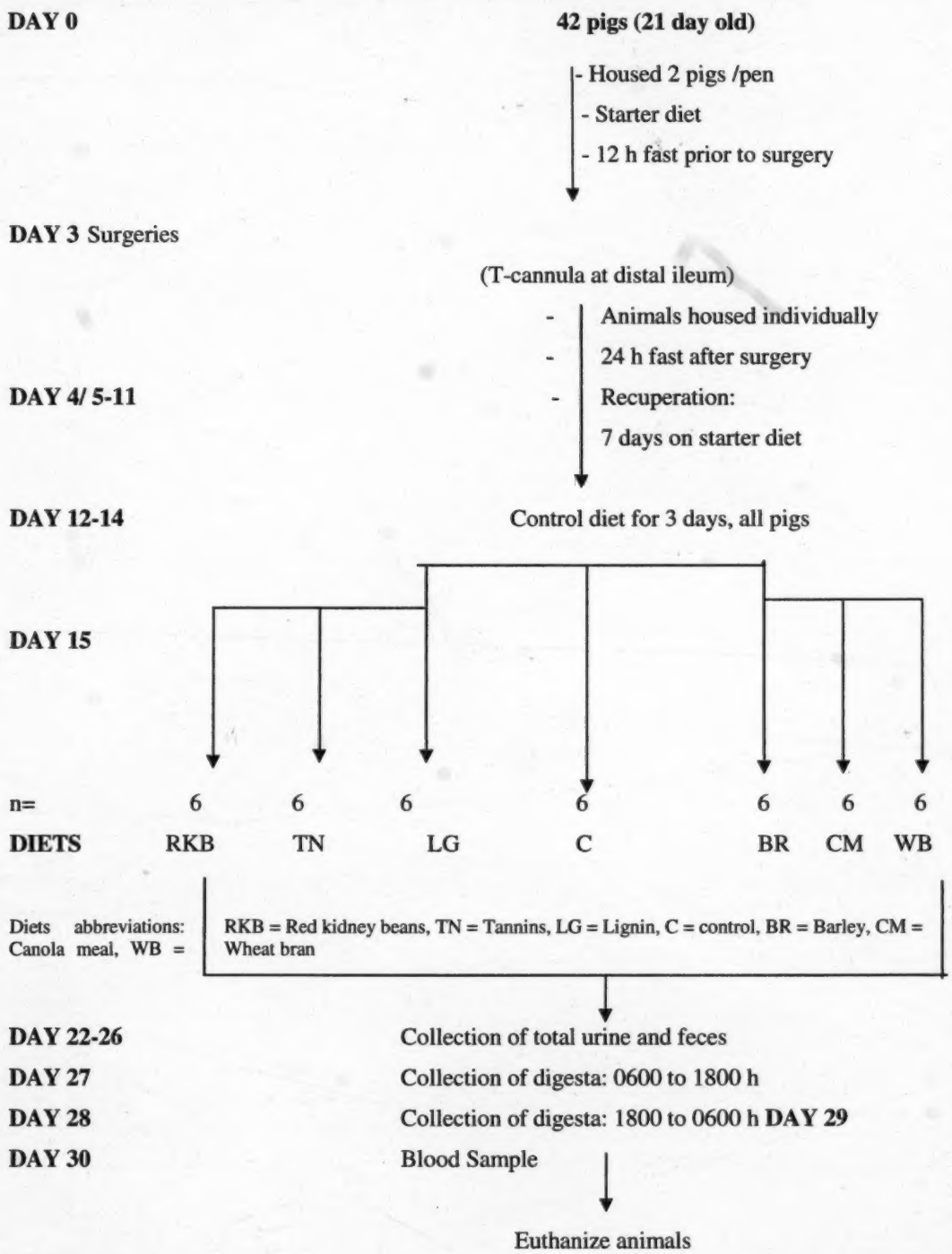
tannins, fibre components) in common pig feedstuffs increase the output of mucin protein from the small intestine, and subsequently result in an increase threonine loss.

4.2 Materials and Methods

4.2.1 Experimental Design

Below is a schematic (Figure 4.2.1) of the experimental design, which is explained in detail in the following section of this chapter. Protocols and procedures for this study were approved by the Animal Policy and Welfare Committee of the Faculty of Agriculture, Forestry and Home Economics, at the University of Alberta.

Figure 4.2.1: Experimental design for feeding growing pigs ANF-based diets


DAY 0
42 pigs (21 day old)
- Housed 2 pigs /pen
- Starter diet
- 12 h fast prior to surgery
DAY 3 Surgeries
(T-cannula at distal ileum)
- Animals housed individually
- 24 h fast after surgery
DAY 4/ 5-11
- Recuperation:
7 days on starter diet
DAY 12-14
Control diet for 3 days, all pigs
DAY 15
n= 6 6 6 6 6 6 6
DIETS RKB TN LG C BR CM WB
Diets abbreviations: Canola meal, WB =
RKB = Red kidney beans, TN = Tannins, LG = Lignin, C = control, BR = Barley, CM = Wheat bran
DAY 22-26
Collection of total urine and feces
DAY 27
Collection of digesta: 0600 to 1800 h
DAY 28
Collection of digesta: 1800 to 0600 h DAY 29
DAY 30
Blood Sample
Euthanize animals

4.2.2 Animals and Surgery

Forty-eight pigs were obtained from the University of Alberta Swine Research and Technology Center at 21 days old (6-7 kg). Young growing pigs were used because their small intestines are expected to be more sensitive to mucus-stimulating ANF (Huisman & Tolman 1992). On arrival, animals were fed a commercial starter diet, (see Table 4.2.1) for 2 to 3 days before surgery. During this period, animals were housed in groups of two or three. Surgery (adapted from Sauer et al. 1977) was performed three days after arrival. The animals were fasted for 12 h prior to surgery. Long-acting Liquamycin (0.5ml; Rogar/STB Inc., London, ON), was administered intramuscularly 8 h before surgery. One-half hour before surgery, the pigs were tranquilized with Atravet (Ayerst Veterinary Labs, Guelph, ON). The pigs were brought under general anaesthesia using a gas mixture of halothane (Ayerst Laboratories, St. Laurent, PQ) and oxygen. The right side of the pig, starting from the second last rib caudally across the flank region, was shaved with an electric clipper, cleaned with Betadine (Purdue Frederick Inc., Toronto, ON) and draped leaving the surgical area exposed. An incision approximately 7 cm long was made parallel and caudal to the last rib leaving a space of approximately 3 cm between the incision and the last rib. The distal ileum was identified by locating the ileo-caecal junction. The incision site in the ileum was marked 5 cm cranial to the ileo-caecal junction. Catgut, 2-0, (Daviv & Geck Cyanamid Canada Inc., Baie D'urfe, PQ) was positioned by aid of a purse-string suture through the serosal layer of the intestine to outline the positioning and length of the incision. A scalpel blade was used to make an incision between the two parallel sutures. After the flanges of the cannula were inserted into the incision, the suture was tightened. Approximately 2 mm below this suture, another purse-string suture was placed around the base to further secure the cannula. Extruded mucosa was trimmed with a scalpel blade. A fistula was created between the last two ribs by surgically removing a small piece of skin and penetrating the muscle layers and peritoneum using finger manipulation and a pair of Rochester Pean Forceps. The barrel of the cannula was then pulled through the fistula. The retaining ring was fixed as close as possible to the skin by tying surgical tape around the barrel of the cannula. Terramycin (0.5 ml; Dominion Vet. Laboratories Ltd., Winnipeg, MB), was administered into the abdominal cavity before the incision was closed. The cannulas were made to be approximately 3 mm thick, with internal diameter of the barrel of the cannula about 13 mm; the wings were 16 mm in width and 40 mm length. The pigs were returned to their respective metabolic crates after surgery. Pigs recovered from anaesthesia within 2 to 3 h of surgery. The animals were fasted for 24 h after surgery.

The next day (24 h post surgery), the pigs were provided with 25 g of the starter diet four times daily at 6 h intervals: 0600, 1200, 1800 and 0000 h, respectively. The daily dietary allowance was gradually increased until all the pigs consumed the starter diet at a rate of 5% of their body weight (BW); which occurred within 7 days. During the recuperation period, the crate temperature was maintained at 30 to 32 ^{0}C by adjusting the positioning of the infrared heating lamp. On days 1, 2 and 3 after surgery, Torbugesic (0.05 ml/kg BW) (Wyeth Animal Health Canada, Guelph, ON) was administered intramuscularly. From day 3 post-surgery onwards, the animals were washed with warm water twice daily around the cannula area and zinc oxide (Coopers Agropharm Inc., Ajax, ON) applied under and around the retaining ring to minimize skin irritation.

4.2.3 Diets

Pigs consumed the starter diet (Table 4.2.1) for a total of twelve days. Forty-two pigs were selected based on feed intake and adapted to the casein-based control diets for three days. Pigs (mean weight = 9.45 kg; SEM = 0.11) were then randomly allocated to either the control diet or one of six test diets in a complete randomized design. Test diets were casein-based plus: lectins (red kidney beans), tannins, barley, wheat bran, canola meal, and lignin (see Table 4.2.2). All seven diets were similar in terms of ingredients, except some adjustments were made to ensure nutrient contents of all diets met the requirement of the pigs. All diets were formulated using NRC Nutrient Requirements of Swine (1998), to be isonitrogenous and isocaloric (Table 4.2.3). Table 4.2.2 shows the diets compositions and Table 4.2.3 to Table 4.2.6 shows nutrient compositions of the diets. Below are brief explanations for the level of each test ingredient included. The aim was to supplement the diets with these ingredients at levels that are normally found in standard pig diets.

Lectins (red kidney beans): Pusztai et al. (1975, 1977) fed rats a purified preparation of lectins from *Phaseolus vulagaris* (red kidney beans = RKB) and found that 0.1% and higher caused a definite inhibition of growth and 0.5% lectins caused the death of the animals within 2 weeks. The level of lectins that would be found in typical pig diets containing feedstuffs such as canola meal, wheat, barley and other cereals (see Nachbar et al. 1980 for lectins content of foods) is approximately 0.30% bean lectins (15 g RKB); this level also represents a safe level within the linear growth inhibition range of Pusztai's graphs. Unico dried red kidney beanss (Unico Inc., Concord, ON) were used in this study. The beans were ground through a 1.0 mm mesh screen in a model 4 laboratory Wiley mill (Wiley Arthur Thomas Co., Philadelphia, PA).

Tannins: Literature reviews suggest that the levels of condensed tannins in plants that may impact pig performance are in a range from 1.4% to 3.8% (Cousins et al. 1981, Jansman 1993). At the higher tannins levels the inclusion levels of the plant sources were as high as 90%. However, this inclusion level for such feeds is not the normal practice in a pig-feeding situation. Thus, for this study, a tannins level of 2% was chosen because it more realistically represents the inclusion levels from plant sources that may be fed to pigs (see Bravo 1998 for tannins content of foods). Grape tannins (Prescott & Co., Mississauga, ON) was used in this study.

Barley: Most pigs in Western Canada are fed barley (Patience et al. 1995). Barley can constitute as much as 80% of a pig's diet. Because of barley's high fibre content, the amounts offered to young pigs are usually limited because it may have a negative impact on their growth and development. An inclusion level of 15% of the diet was chosen because it facilitated comparison to the canola meal and wheat bran test diets. Both canola meal and wheat bran were included at levels usually found in young pig diets (see below). The barley, canola meal and wheat bran diets were formulated to provide comparable NDF, ADF and hemi-cellulose contents. The barley used for this study was six-row barley, obtained from the University of Alberta feed mill, 2001 harvest.

Canola meal: Canola meal at up to 12% of the diet has been shown to be acceptable for starting, growing and finishing pigs (Patience et al. 1995). An inclusion level of 10% canola meal was used because barley, canola meal and wheat bran diets were formulated to provide comparable NDF, ADF and hemi-cellulose contents. Canola meal for this study was obtained from the University of Alberta feed mill, 2001 harvest.

Wheat bran: An inclusion level of 10% was used based on other research in this area (see Chapter 2), and for similarity to NDF, ADF, and hemicellulose contents with barley and canola meal diets. Rogers 100% natural wheat bran (Rogers Foods Ltd., Armstrong, BC) was used in this study.

Lignin: Shah et al. (1982) observed that lignin at levels of 3% and 6% in rat diets caused a decrease in body weight, an increase in excretion of fecal nitrogen, and a significant decrease in net protein retention (NPR). Lignin levels of 3-6% are normally present in most food sources (Chiou et al. 1994, Shah et al. 1982). In keeping with the levels of lignin in barley, canola meal and wheat bran (see Table 2.3.1) and with the levels in most natural foods (Shah et al. 1982), an

inclusion level of 3% lignin in the diet was chosen. Tembec Chemical & Power Group Inc., (Temiscaming, Quebec, Canada) generously supplied the lignin (sodium lignosulphonate) that was used in this study.

Table 4.2.1: Nutrient and energy content of the commercial starter diet for weaned pigs[1]

Diet Nutrient	Diet Amount
Crude protein	24.46 %
Digestible energy	3.61 Mcal/kg
Calcium	0.97 %
Phosphorus	0.77 %
Lysine	1.66 %

[1]Consultant Feeds, Calmar, Alberta, Canada, produced this diet. In keeping with Competitive Privacy Act, the above information is the only information that they will divulge. Pigs received this diet for 12d following weaning.

Table 4.2.2: Experimental diet composition (g/kg) as formulated using NRC (1998)

	Test Diets						
Ingredients	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins
Barley	0	0	0	150	0	0	0
Canola Meal	0	100	0	0	0	0	0
Casein	145	123	140	140	145	142	145
Corn oil	4.0	22.5	34.0	38.0	24.0	2.0	17.5
Cornstarch	380.78	312.69	274.60	212.59	338.28	376.04	352.28
Chromic oxide	5	5	5	5	5	5	5
Di-Calcium-phosphate	15	15	15	15	15	15	15
Potassium chloride	5.25	3.00	3.00	4.00	5.25	5.00	5.25
Kidney beans	0	0	0	0	0	15	0
L- Asparagine	48	40	44	44	48	48	48
L-Cystine	2.75	2.10	2.50	2.50	2.75	2.75	2.75
Lignin	0	0	0	0	22.5	0	0
Limestone	10	10	10	10	10	10	10
L-Threonine	0.9	0.5	0.7	0.7	0.9	0.9	0.9
L-Tryptophan	0.3	0.2	0.2	0.2	0.3	0.3	0.3
Magnesium sulphate	2	0	0	2	2	2	2
Sodium bicarbonate	6	6	6	6	6	6	6
Sugar	350	350	350	350	350	350	350
Solkafloc[a]	15	0	5	10	15	10	15
Tannins	0	0	0	0	0	0	15
Thiamine	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin B_6	0.01	0.01	0	0	0.01	0.01	0.01
Vitamin-Mineral mix [b]	10	10	10	10	10	10	10
Wheat bran	0	0	100	0	0	0	0
Sum	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

[a]Provided cellulose.[b]Provided the following per kg of vitamin mix: 21.5 g Ca; 8.4 g P_{dig}; 1 g Mg; 0.20 g S; 595 mg Cu; 9.9 mg I; 7 g Fe; 1.6 g Mn; 7.4 mg Se; 3.5 g Zn; 15mg biotin; 13 g choline; 80 mg folacin; 625 mg pantothenic acid; 950 mg niacin; 175 mg riboflavin; 880 ug vit B_{12}; 1550 mg vit. E; 300,000 IU vit. A; 35,000 IU vit. D. Kidney beans were used as a source of lectins (Unico Inc., Concord, ON). Grape tannins was used as a source of tannins (Prescott & Co., Mississauga, ON). Wheat bran was from Rogers Foods Ltd, Armstrong, BC. Lignin used was sodium lignosulphonate from Tembec Chemical & Power Group Inc., Temiscaming, PQ. Barley and canola meal were obtained from the University of Alberta feed mill, 2001 harvest.

Table 4.2.3: Nutrient and energy content of diets in Experiment 1, calculated

		Test Diets						
Nutrient	Units	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins
Ash (minerals)	%	4.92	5.16	5.30	5.10	4.92	4.93	4.92
Crude Protein	%	19.63	20.07	20.15	20.14	19.62	19.70	19.62
Dry matter	%	95	95	95	95	95	95	95
Crude Fat (ether extract)	%	0.24	1.37	2.19	2.38	1.42	0.12	1.04
ME	Kcal/kg	3621	3628	3626	3626	3621	3621	3621
ME	MJ/kg	15.16	15.19	15.18	15.18	15.16	15.16	15.16

ME = Metabolizable energy, calculated from digestibility energy. ME is expressed as KJ/kg and Kcal/kg. KJ/kg = 238.85 Kcal/kg.

Table 4.2.4: Nutrient and energy content of diets in Experiment 1, analyzed

		Test Diet						
Nutrient	Units	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins
Ash	%	4.40	4.78	4.68	4.53	4.98	4.22	4.21
Crude Protein	%	17.80	18.96	18.06	19.27	18.30	18.08	17.81
Dry matter	%	94.65	94.94	94.70	94.71	94.83	94.65	94.52
Ether extract	%	0.19	1.89	2.85	2.75	1.79	0.12	1.13
GE[1]	Kcal/kg	3830	4018	4072	4339	3951	3844	3939
DE[2]	Kcal/kg	3686	3658	3292	3856	3195	3662	3565
ME[3]	Kcal/kg	3578	3540	3187	3730	3097	3551	3454
ME[3]	MJ/g	14.98	14.82	13.34	15.62	12.96	14.87	14.46

[1]GE = Gross energy, determined by bomb calorimeter. [2]DE = Digestibility energy. DE was calculated using the determined apparent energy for the diets multiplied by the GE values of the diets. Apparent E = [1- (ND * CrF) / (NF * CrD)] * 100%, and DE = apparent digestible E*GE of diet treatment [3]ME = Metabolizable energy, calculated from digestibility energy. ME is expressed as KJ/kg and Kcal/kg. KJ/kg = 238.85 Kcal/kg. The average of Equation 4.2.1, Equation 4.2.2, Equation 4.2.3 were used to determine ME.

Table 4.2.5: Fibre content (g/kg DM) of Experiment 1 test diets, analyzed

Component	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins
NDF	26.41	64.07	78.72	98.89	24.89	23.89	24.92
NDR	32.08	73.24	85.75	107.08	31.96	27.82	30.79
ADF	5.45	17.17	17.65	19.65	8.14	3.66	5.06
HC	20.96	46.30	61.07	79.24	16.75	20.23	19.85
Cellulose	14.23	18.81	14.99	17.67	5.15	5.34	6.06
TC	35.19	65.11	76.05	96.92	21.90	25.57	25.91
Lignin	-0.56	5.86	3.91	5.36	3.54	0.05	-0.95

NDF = neutral detergent fibre, NDR = neutral detergent residue, ADF= acid detergent fibre, HC= hemicellulose, TC= total cellulose, i.e., HC and cellulose

Table 4.2.6: Amino acid composition of diets (% in the diet) [1]

	Test Diets						
Amino acids	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins
Alanine	3.58	4.55	4.08	4.24	3.58	3.64	3.58
Arginine	3.61	5.10	4.45	4.39	3.61	3.73	3.61
Aspartate	8.50	9.72	9.18	9.33	8.50	8.71	8.50
Cystine	1.00	1.64	1.27	1.40	1.00	1.02	1.00
Glutamic acid	26.78	28.68	28.64	30.70	26.78	26.75	26.78
Glycine	2.20	3.61	2.91	2.85	2.20	2.29	2.20
Histidine	3.16	3.56	3.43	3.44	3.16	3.19	3.16
Isoleucine	6.28	6.63	6.47	6.71	6.28	6.30	6.28
Leucine	11.77	12.44	12.22	12.79	11.77	11.80	11.77
Lysine	9.21	9.59	9.40	9.51	9.21	9.24	9.21
Methionine	3.78	3.91	3.86	4.00	3.78	3.75	3.78
Phenyl-alanine	6.12	6.61	6.48	6.93	6.12	6.18	6.12
Proline	13.43	13.45	13.91	14.99	13.43	13.28	13.43
Serine	6.63	7.14	7.00	7.24	6.63	6.68	6.63
Threonine	6.41	6.68	6.47	6.67	6.41	6.43	6.41
Tryptophan	2.00	2.11	2.12	2.06	2.00	2.00	2.00
Valine	7.79	8.30	8.14	8.42	7.79	7.80	7.79
Sum	122.25	133.71	130.03	135.65	122.25	122.79	122.25

[1]Degussa AG, Germany, performed amino acid analyse on diet samples (Llames & Fontaine 1994).

4.2.4 Study Protocol

Following a 3d adaptation to control diet, the experimental diets were fed for 16 days. During the adaptation and test diet periods, the animals' daily feed allowance were divided into two equal portions and provided at 0600 h and 1800 h. Water was freely available from a low-pressure drinking nipple. The room was lit from 0600 h to 1800 h. Individual feed intakes and body weights were recorded daily to determine average daily gain and feed efficiency.

Following 7 days adaptation to the test diets, total urine and feces were collected for 5 days, in separate 24 h periods, from individual pigs to determine nitrogen balance, fibre and protein digestibility. Concentrated sulphuric acid (from 5 ml to 8 ml) was added to each urine flask to prevent ammonia loss. Following collection, aliquots of urine and feces were frozen at -20 ^{0}C for later analyses. Ileal digesta was collected for a total of 24 h, from 0600 h to 1800 h on day 27 and from 1800 h on day 28 to 0600 h of day 29, for determination of mucin and for other analyses. For the collection of ileal digesta, soft plastic tubing containing 10 to 15 ml formic acid was secured to the barrel of the cannula with twist-tie. The plastic tubing was removed and replaced when filled with digesta. Digesta was stored at -20 ^{0}C until analyzed. The digesta was freeze-dried, pooled within pig, ground through a 0.8 mm mesh screen in a model 4 laboratory Wiley mill (Wiley Arthur Thomas Co., Philadelphia, PA) and mixed prior to analysis.

4.2.5 Blood Sampling and Intestinal Collection Procedures

After the ileal digesta collection period, animals were sedated using halothane and oxygen. Twenty ml of blood was obtained from the heart via needle puncture and then transferred into heparinized tubes, centrifuged and plasma samples collected and frozen at -80 ^{0}C until analyzed for amino acid concentrations.

The entire small intestine and large intestine were then excised, rinsed in ice-cold physiological saline solution (0.9% NaCl) and, blotted dry. Weight and length measurements were performed on the duodenum and small and large intestines. Tissue samples (~2 cm) of the duodenum, jejunum, ileum, and colon were frozen immediately with liquid nitrogen, and kept at -20 ^{0}C for protein and amino acid determination. The duodenum (from the stomach to the duodenojejunal flexure) and approximately 60 cm sections of the ileum, mid- jejunum, distal-jejunum and 80 cm sections of the colon were weighed, cut open and rinsed of digesta contents. The mucosa was

removed from the tissue samples by gently scraping with a glass microscope slide. The mucosa was weighed, immediately frozen in liquid nitrogen, and stored at -20 ^{0}C for further analyses. The remaining muscularis mucosa was also weighed and frozen in liquid nitrogen, then stored at -20 ^{0}C for further analysis. The liver, kidney, spleen, and emptied stomach were also removed, blotted dry and weighed. Pigs were then killed by given 0.2 ml/kg BW of sodium pentobarbital via intra-cardiac injection.

4.2.6 Analyses

4.2.6.1 Fat, Energy, Ash, and Analytical Dry Matter

These analyses were performed in duplicate according to AOAC (1984) methods.
Analytical dry matter was determined for feed, feces and digesta samples. Samples (2 g each) were weighed and dried in an oven at 110 ^{0}C for 5 h then re-weighed post drying. Ash analysis was also performed on these samples by placing samples in an oven at 550 ^{0}C overnight and the residual weighed.

Gross energy content of feeds and feces were determined using a Leco Bomb Calorimeter (AC-300, Leco Corp., St. Joseph, MI). The procedure involved placing approximately 1 g of pellet sample in a special metal container. Then 10 cm of platinum wire was attached to the wire holder, with the wire touching the surface of the sample. The unit, consisting of the sample and wire, was then placed inside the bomb unit, which was then tightly capped and filled with 440 psi oxygen. The unit was then submerged in water and combustion was allowed to occur for approximately 12 min. Gross energy of the sample was determined from the change in temperature of the water. For the experimental diets, the average of the following three equations was used to determine ME:

Equation 4.2.1: ME (May & Bell 1971):

ME= DE (1.012 – (0.0019*%CP)

Equation 4.2.2: ME (Noblet et al. 1989):

ME = DE (0.988 – (0.002*%CP)

Equation 4.2.3: ME (Noblet & Perez 1993):

ME = DE (1.003 – (0.0021*%CP)

Crude fat (fat ether) was determined using a Goldfisch extraction apparatus (Labconco Co., Kansas City, MO). The procedure involved measuring approximately 40 ml petroleum ether into an extraction beaker, and weighing approximately 2 g of dried sample on an appropriate grade filter paper, and then placed in a sample holder. The sample holder and the extraction beaker were attached to the Goldfisch unit; the heat was set to high and the condenser water unit turned on and up to 6 h of extraction allowed to occur. Following the extraction, once all the petroleum ether had evaporated from the beaker, it was placed in a 110 ^{0}C oven for 30 min. The beaker was cooled to room temperature in a dessicator, and weighed. Fat content was determined by the following equation:

Equation 4.2.4: % Fat

= 100 x [(wt. beaker + wt. extract – wt. blank residue) – (wt. beaker)]/ wt. sample

4.2.6.2 Nitrogen Analysis

Nitrogen contents of feed and digesta samples were determined by the method described by Sweeney and Rexroad (1987). This method involves sample combustion in an oxygen atmosphere followed by a series of catalytic reactions resulting in nitrogen gas production. Samples (100 mg portions) were weighed into tin foil and wrapped. Samples were then analyzed using a LECO FP-428 Nitrogen/Protein Determinator (LECO Instruments Ltd., Mississauga, ON).

Nitrogen contents of feces and urine samples were determined by Kjeldahl analysis (Bradstreet 1965). Aliquots of urine (5 ml samples) or 2 g samples of feces were placed into a 500 ml flask and a catalyst pack containing 9.9 g K_2SO_4, 0.41 g HgO and 0.08 g $CuSO_4$, was added. Concentrated H_2SO_4 (30 ml) was added, and the samples were digested for 30 minutes at high temperature and then cooled to room temperature. Water (300 ml) was added to the flask and the solution was thoroughly mixed. Approximately 1 g of Zn metal (20 mesh) was added and mixed, followed by the addition of 100 ml of 40% NaOH; the solution was then swirled and heated. The resulting distillate about 125 ml was collected in 250 ml flasks containing 50 ml of 5% boric acid (containing bromocresol green and methyl red indicators). The ammonia was then titrated with 0.1 N H_2SO_4 to an end point of a light pink colour. For conversion to protein content, the following calculation was used:

Equation 4.2.5: % Protein

= % N x 6.25,

Equation 4.2.6: %N

$$= \frac{[(\text{ml of } H_2SO_4 - \text{blank}) \times \text{Mol. wt. of N} \times \text{Normality of } H_2SO_4 \times 100\%]}{\text{Mass of sample}}$$

4.2.6.3 Fibre Analysis

Fibre analysis was performed using a modified method of Goering and van Soest (1970). This method is known as the filter bag technique (FBT) (Ankom Co. publication # 101, 1993). Analysis of NDF and ADF by the FBT was essentially the same as the conventional technique, except for the way the samples were filtered. The filtering procedure started at the detergent solubility step and continued through the rinse steps. The procedure involved 0.5 g of sample placed in a filter bag (Ankom Co., Fairport, NY) and the bag heat-sealed. Up to 24 bags were put into a special bag suspender (Ankom Co., Fairport, NY) and placed in NDF or ADF detergent solutions held at 98 ^{0}C for 70 min for NDF and 60 min for ADF. The NDF solution consisted of 1.08 g sodium lauryl sulfate, USP; 670 g Ethylenediaminetetraacetic Disodium Salt, Dihydrate; 245 g sodium tetraborate decahydrate; 164 g sodium phosphate dibasic, anhydrous; and 360 ml triethylene glycol, in 36 L distilled water, pH range of 6.9 to 7.1. The ADF solution consisted of 360 g cetyl trimethylammonium bromide (CTAB), 500 ml sulfuric acid (72% by weight) and 17.5 L distilled water. The bag suspender was used to transfer the bags through a series of four hot water rinses (near boiling) and a final acetone rinse. The filter bags were first air-dried and then placed in a 110 ^{0}C oven for at least 4 hours. Samples were placed in a dessicator to equilibrate to room temperature and weighed. NDF, ADF and lignin assays were performed on the same sample. For lignin assay the bags with the samples were placed in 72% H_2SO_4 for 3 hours, with gentle agitation every half hour. Samples were rinsed in hot water until pH was neutral and then rinsed in acetone. Drying procedures were the same as used for ADF and NDF. Fibre content was determined by the following equation:

Equation 4.2.7: % Fibre

= [(Wt. Bag with fibre – filter bag wt.)/ Sample wt.] x 100

4.2.6.4 Amino Acid Analysis of Diet and Digesta

Amino acids were determined in diets by Degussa AG (Hanau, Germany) and in ileal digesta by high performance liquid chromatography (HPLC) following acid hydrolysis in 6 N HCl for 24 h (Sedgwick et al. 1991). Approximately 100 mg of freeze-dried digesta or 50 mg of feed was weighed into 10 ml screw cap test tubes. Six ml of 6 N HCl was added and then the tubes were purged with nitrogen and the samples hydrolysed at 110 ^{0}C for 24 h. Following hydrolysis, 1 ml of water and 200 µL of internal standard were added. Internal standards contained both β-amino-n-butyric acid and ethanolamine at 25 µmol/ml in water. Amino acid standards were prepared by combining 1 ml of Sigma amino acid standard (2.5 µmol/ml, Sigma Chemical Co., St. Louis, MO), 200 µl internal standard and 6 ml 6 N HCl in a screw cap test tube. Amino acids were analyzed as O-phthaldialdehyde derivatives using HPLC. Methionine, cysteine/cystine and proline were not determined for the digesta samples.

4.2.6.5 Plasma Amino Acid Analysis

Plasma amino acid concentrations were measured using reversed- phase HPLC using phenylisothiocyanate (PITC) derivatives. Plasma (200 µL) was combined with 40 µL of 2.5 µmol/ml internal standard norleucine, and 1 ml of a precipitation solution (0.5% trifluoroacetic acid in methanol). After vortexing, the protein was removed by centrifugation at 1500 x g for 5 min and samples were freeze-dried overnight. A 50 µL solution of triethylamine, methanol and water at a concentration of 1:1:3, respectively, was added and the sample freeze-dried to dryness. The amino acids were derivatized for 35 min, with 20 µL of a 1:1:7:1 solution of water, triethylamine, methanol and phenylisothiocyanate, respectively, and then freeze-dried overnight. The amino acid derivatives were re-suspended in 200 µL of a solution containing 95% phosphate buffer (5 mM Na_2HPO_4 adjusted to pH 7.4 with 10% phosphoric acid) and 5% acetonitrile. Samples were centrifuged at 1500 x g for 5 min. Aliquots (10 µL portions) of the derivatized samples were injected onto a C-18 reversed-phase column maintained at 46 ^{0}C and eluted using a convex binary gradient. Buffer A contained 70 mM sodium acetate and 2.5% acetonitrile, adjusted to pH 6.55 with concentrated acetic acid. Buffer B consisted of a 45:40:15 ratios of acetonitrile, water and methanol, respectively. Amino acid derivatives were detected at a wavelength of 254 nm and processed through a data station.

Amino acid concentration was calculated as follows:

Equation 4.2.8: AA (µmol/L)

= (AA peak area/ norleucine peak area)* CF *RF

Where, CF is concentration factor (250 µmol/L)

RF is response factor, which is norleucine peak area/AA peak area.

4.2.6.6 Digestibility Determinations

Chromic oxide analyses were performed by the method of Fenton and Fenton (1979) and apparent digestibility of protein and energy in feces and ileal digesta were determined by the following equation:

Equation 4.2.9: %Apparent fecal and ileal digestibility

$= [1- (N_D * Cr_F) / (N_F * Cr_D)] * 100\%$

Where, N_D is the nutrient concentration in feces or ileal digesta,

Cr_F is Cr_2O_3 dietary concentration,

N_F is nutrient concentration in the diet,

Cr_D is Cr_2O_3 fecal or ileal digesta concentration

Fecal samples containing 20-60 mg Cr_2O_3 (1 g sample) were ashed overnight at 550 ^{0}C. Digestion mixture (15 ml portions), containing 10g of sodium molybdate dihydrate dissolved in 500 ml of a 15:15:20 mixture of water, concentrated sulphuric acid and 70% perchloric acid, respectively, was added to each sample. Samples were heated on a hot plate until a yellowish or reddish colour developed. After cooling, samples were diluted to 100 ml with water, centrifuged (1000 x g) for 5 min and the absorbance read at 440 nm (Milton Roy Spectronic 3000 Array, Milton Roy Co., Rochester, NY).

Ileal nutrient flow was calculated as described by Furuya & Kaji (1992). The flow of nutrient through the terminal ileum was calculated using Cr_2O_3 in the diet. The equation is as follows:

Equation 4.2.10: Ileal nutrient flow (g/d)

= (ileal nutrient (g/g)) * [diet Cr_2O_3 (g/g)/ileal Cr_2O_3 (g/g)] * (feed intake (g/d)

4.2.6.7 Mucin Analyses

4.2.6.7.1 Digesta - Isolation of Crude Mucin

Crude mucin was isolated according to modifications (by Lien 1995) of procedures described by Allen (1981). Approximately 3 g of freeze-dried digesta were weighed into a 50 ml polystyrene test tube and 25 ml of 0.15 M NaCl containing 0.02 M sodium azide (maintained at 4 ^{0}C), was added and samples were homogenized for 1 min using a Polytron homogenizer (Kinematica, Kriens, Switzerland). Homogenized samples were immediately centrifuged for 30 min at 12,000-x g at 4 ^{0}C and the aqueous layer decanted into a second 50 ml polystyrene test tube. The aqueous layer was again centrifuged for 30 min at 12,000-x g at 4 ^{0}C to ensure the complete removal of insoluble material. Sixteen ml of the aqueous layer were pipetted into a pre-weighed 50 ml test tube, cooled in an ice-bath, and ice-cold ethanol added to a final concentration of 60% (v/v). The samples were allowed to precipitate overnight at –20 ^{0}C.

The following day samples were centrifuged at 1400-x g for 10 min at 4 ^{0}C and the precipitate recovered by decanting the supernatant. The pellet was resolubilized in 16 ml of 0.15 M NaCl and cooled in an ice-bath. Pre-cooled ethanol was added to a final concentration of 60% (v/v) and the samples were left overnight to precipitate as before. The crude mucin precipitate was recovered as described in the paragraph above. Successive rinsing resulted in reduced recovery of crude mucin, without any change in the composition; therefore, samples were rinsed only until a clear supernatant was obtained. The precipitate was resolubilized in 10 ml of water and freeze-dried. Recovery of crude mucin from digesta was linear between 0.5 and 8 g of starting material.

4.2.6.7.2 Crude Mucin – Amino Sugar Analysis

Amino sugars were analyzed as their alditol acetates according to procedures of Lien (1995) adapted from Blakeney et al. (1983) and Kraus et al. (1990). Approximately 50 mg of CM or ileal digesta were treated with 12 M sulphuric acid (1.5 ml) for 1 h at room temperature. The solution was diluted to 3 M with 4.5 ml of water and the samples hydrolyzed for 1 h at 110 ^{0}C. Following hydrolysis, 200 µL of internal standard were added (N-methylglucamine and *myo*-inositol, for amino sugars and neutral sugars, respectively, at 10 mg/ml of distilled water). Aliquots (1 ml) of the acid hydrolysates were cooled in an ice-bath and made basic with the addition of 0.7 ml concentrated ammonium hydroxide. To 100 µL of this, 1 ml sodium borohydride (30 mg/ml in anhydrous dimethylsulphoxide) was added and the reduction reaction was allowed for 90 min at 40 ^{0}C. Excess sodium borohydrate was decomposed with the addition of 200 µL concentrated glacial acetic acid. Following this, 0.2 ml 1-methylimidazole and then 2 ml acetic anhydride were added. The solution was mixed and acetylation occurred at room temperature for 10 to 15 min.

Thereafter, 5 ml of water was added to decompose excess acetic anhydride and the mixture cooled to room temperature. Alditol acetates were extracted into 4 ml of dichloromethane by vigorous shaking and the upper aqueous layer removed. The dichloromethane layer was rinsed twice with 4 ml of water and evaporated to dryness under a stream of nitrogen. Standard sugars and derivatization reagents were obtained from Sigma (Sigma Chemical, St. Louis, MO).

Prior to analysis by gas-liquid chromatography (Varian 3400), the alditol acetates were re-dissolved in 1 ml of dichloromethane and approximately 0.5 µL of derivatized sample was injected onto a DB-17 fused silica capillary column (0.25 mm i.d. x 30 m; J&W Scientific, Folsom, CA). Helium was used as the carrier gas at a rate of 1.5 ml/min. Injector temperature was programmed from 60 ^{0}C to 270 ^{0}C at 150 ^{0}C /min and maintained for 20 min. Oven temperature was raised at 30 ^{0}C /min from 50 ^{0}C to 190 ^{0}C, held for 3 min, then increased 5 ^{0}C /min to 270 ^{0}C and held for 5 min. Detector (flame ionization) temperature was set at 270 ^{0}C.

Calculations

Regression equations were derived from the N-acetylglucosamine (GlcNAc) to N-acetylgalactosamine (GalNAc) ratios in purified gastric (Scawen & Allen 1977) and intestinal (Mantle & Allen 1981, Mantle et al. 1981) mucins to calculate the contributions of gastric mucin and the GalNAc content. Two formulas were derived, assuming complete native (no proteolytic digestion) or proteolytic (pronase) digested mucin, to estimate the range of mucin output. The relationship between the GlcNAc\ GalNAc ratio and contribution of gastric mucin is described by the following regression equations:

Equation 4.2.11: % Native gastric mucin

$$= -80.23 + 183.26x - 71.19x^2 + 11.05x^3$$

Equation 4.2.12: % Pronase gastric mucin

$$= -82.07 + 188.36x - 74.50x^2 + 11.69x^3$$

x = GlcNAc/ GalNAc ratio. The GalNAc content of mucin mixtures is described by the following regression equations:

Equation 4.2.13: % Native GalNAc

$$= 32.30 - 22.74x + 8.83x^2 - 1.37x^3$$

Equation 4.2.14: % Pronase GalNAc

$$= 34.87 - 25.36x + 10.03x^2 - 1.57x^3$$

x = GlcNAc/GalNAc ratio. Daily output of mucin was calculated from the estimate of GalNAc content and daily outputs of GalNAc in CM or ileal digesta by the following equation:

Equation 4.2.15: Mucin output

$$= \text{GalNAc} / \%\text{GalNAc}$$

where GalNAc = GalNAc output in g /day

Crude mucin flow was calculated as followed:

Equation 4.2.16: CM flow

$= (Cr_{diet} / Cr_{digesta}) * cdM_{digesta} * 1000$ (Adapted from Montagne et al. 2000). Cr_{diet} and $Cr_{digesta}$ are chromic oxide in the diet and ileal digesta, respectively; $cdM_{digesta}$ is crude mucin concentration in ileal dry matter (DM) digesta.

4.2.7 Statistical Analyses:

The experiment used complete randomized design with seven treatments and six replicates. Calculations given by Berndtson (1991) show that this sample size would enable detection of a 20% difference from control treatment with CV = 9%, $\alpha = 0.05$ and a power of 90%. Data were analyzed using the General Linear Model procedure of Statistical Analysis System (SAS System version 8, 1999). Differences between treatments for average daily gain, nitrogen balance, mucin output, and digesta and plasma amino acid concentrations were assessed by protected LSD multiple comparisons procedure and were considered statistically significant at $p < 0.05$. Analysis of covariance (ANCOVA), using initial weight as the covariate was tested against weight gain and was non-significant.

4.3 Results

4.3.1 Mucin Output

4.3.1.1 Crude Mucin

The expression of crude mucin per percentage of ileal digesta (g DM /g DM), showed the highest values ($p < 0.01$) for lignin and control treatments (Table 4.3.1). However, when expressed as crude mucin flow per day (g DM/d) lignin and barley treatments (4.45 and 3.88 g /d, respectively) were significantly higher ($p = 0.04$) than the control treatment (2.60 g/d)

Table 4.3.1: Recovery and daily output of crude mucin (cdM) from ileal digesta samples (dry matter basis) of growing pigs fed various ANF-based test diets

	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Digesta sample (g)	3.01	3.01	2.61	3.00	3.01	2.76	3.01	0.39	0.34
cdM recovered (g)	0.20^{b}	0.12^{c}	0.11^{c}	0.14^{c}	0.26^{a}	0.15^{bc}	0.12^{c}	0.06	<0.0001
cdM/digesta (%)	6.53^{b}	3.99^{d}	4.20^{cd}	4.64^{cd}	8.64^{a}	5.43^{bc}	3.84^{d}	1.94	<0.0001
cdM flow (g/kg DM digesta)	4.36^{c}	4.73^{bc}	6.52^{ab}	6.71^{a}	7.74^{a}	6.00^{abc}	4.23^{c}	2.01	0.01
cdM flow (g/d)	2.60^{c}	2.72^{bc}	3.43^{abc}	3.88^{ab}	4.45^{a}	3.58^{abc}	2.59^{c}	1.21	0.04
cdM increase (%) relative to control	N/A	4.6	31.9	49.2	71.1	37.7	0	-	-

$^{a, b, c}$ means within the same row with different superscripts are significantly different (P<0.05) by LSD multiple comparison test, n=6.

4.3.1.2 Mucin output

The barley-based dietary treatment had the largest mucin output. In comparison to the control treatment all dietary treatments, with the exception of lignin, had greater mucin output. Though numerically smaller, the crude mucin values observed with the use of sugar markers (GlcNAc and GalNAc) (Table 4.3.2) showed similar trends as that for total crude mucin determined in Table 4.3.1, with the exception of lignin. In Table 4.3.2 lignin-treatment produced the least crude mucin flow relative to the control treatment as determined by amino sugars; however, in Table 4.3.1 lignin led to the greatest crude mucin output as determined by precipitation technique.

Table 4.3.2: Mucin outputs[1] from crude mucin and ileal digesta samples from pigs fed ANF-based dietary treatments

	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Crude Mucin (g/day)									
GalNAc	0.78^{c}	4.01^{a}	2.19^{b}	2.70^{ab}	1.62^{b}	3.17^{ab}	2.20^{b}	1.05	<0.001
GlcNAc	0.68	1.61	1.64	1.54	0.77	1.88	1.54	0.46	0.06
Gastric	0.73	0.97	1.28	1.33	0.52	0.98	1.00	0.28	0.21
Intestinal	1.03	1.26	1.43	1.75	0.59	1.37	1.49	0.37	0.43
Total	1.76	2.23	2.71	3.08	1.11	2.35	2.49	0.65	0.20
Ileal Digesta (g/day)									
GalNAc	17.69^{a}	18.69^{a}	22.70^{a}	27.54^{a}	9.33^{b}	22.35^{a}	24.89^{a}	5.96	0.05
GlcNAc	12.77^{ab}	18.79^{a}	23.05^{a}	25.22^{a}	8.75^{b}	18.12^{a}	18.54^{a}	5.65	0.04
Gastric	0.75	1.89	2.11	1.96	1.51	1.84	1.19	0.49	0.19
Intestinal	0.46	1.48	1.31	1.74	1.03	1.99	1.39	0.50	0.06
Total	1.21	3.37	3.42	3.70	2.54	3.83	2.58	0.92	0.13

[1]Determined from the N-Acetylglucosamine (GlcNAc)/ N-Acetylgalactosamine (GalNAc) ratio in crude mucin and the daily output of GalNAc in ileal digesta.

The mucin-carbohydrates, except for N-Acetylgalactosamine (GalNAc), showed similar trend in crude mucin and ileal digesta (Table 4.3.3).

Table 4.3.3: Carbohydrate compositions of crude mucin from ileal digesta of pigs fed various ANF-based diets

	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Crude Mucin (mol/100 mol)									
Fucose	3.11	1.73	1.62	2.41	1.94	2.31	2.18	0.50	0.07
Mannose	0.10	0.08	0.11	0.12	0.06	0.07	0.08	0.02	0.06
Galactose	5.3	3.1	4.4	3.3	2.9	3.9	4.3	0.85	0.11
Glucose	3.61^{a}	2.24^{ab}	2.11^{b}	2.89^{ab}	1.66^{b}	2.64^{ab}	3.80^{a}	0.79	0.04
GlcNAc	1.62	2.31	0.90	2.43	1.42	1.50	2.40	0.59	0.30
GalNAc	3.45	2.22	2.31	3.80	1.56	1.71	1.94	0.87	0.17
GlcNAc /GalNAc	0.75	0.98	0.93	0.92	0.94	0.80	0.71	0.11	0.52
Ileal Digesta (mol/100 mol)									
Fucose	0.50	0.48	0.51	0.46	0.43	0.42	0.58	0.05	0.87
Mannose	0.55	0.47	0.53	0.75	0.57	0.77	0.52	0.12	0.68
Galactose	7.8	11.1	5.2	4.9	5.4	6.0	5.2	2.25	0.45
Glucose	4.09	1.62	1.71	3.42	3.10	2.86	2.42	0.90	0.06
GlcNAc	0.29	0.18	0.14	0.20	0.23	0.31	0.20	0.06	0.64
GalNAc	0.63	0.74	0.42	0.29	0.46	0.42	0.60	0.15	0.13
GlcNAc /GalNAc	0.85	0.55	0.78	0.63	0.61	0.75	0.79	0.11	0.81

N-Acetylglucosamine (GlcNAc) and N-Acetylgalactosamine (GalNAc) ratio in crude mucin and the daily output of GalNAc in ileal digesta

4.3.2 Amino Acid Digestibility

4.3.2.1 Apparent Ileal Digestibility

There were significant differences in the ileal digesta protein contents among dietary treatments. Lignin treatment group had the lowest ileal digesta protein content and the highest was seen in kidney beans treatment group. The amino acid profile was observed to be similar for all dietary treatments; however the amino acid concentrations differ among treatments. The concentrations of alanine, histidine, leucine, lysine and valine were significantly higher (Table 4.3.4) in the digesta samples of wheat bran and tannins treatments compared to control.

Table 4.3.4: Protein[1] (%) and amino acid[2] (mmol AA/g DM digesta) composition of ileal digesta from growing pigs fed various ANF-based diets

Amino acids	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Protein[1]	18.98^{bc}	19.81^{ab}	18.57^{bc}	17.72^{bc}	16.64^{c}	22.51^{a}	19.94^{ab}	3.68	0.03
Alanine	0.644^{b}	0.599^{b}	0.966^{a}	0.720^{ab}	0.463^{b}	0.681^{b}	0.712^{ab}	0.254	0.03
Arginine	0.447	0.497	0.735	0.534	0.392	0.695	0.711	0.243	0.06
Aspartate	1.613	1.442	1.536	1.292	1.011	1.397	1.572	0.801	0.88
Glutamic acid	1.990	1.731	2.047	2.148	1.605	1.907	2.172	0.592	0.58
Glycine	0.720	0.822	0.838	0.820	0.534	0.854	0.877	0.204	0.32
Histidine	0.223^{c}	0.265^{bc}	0.394^{ab}	0.267^{bc}	0.199^{c}	0.325^{abc}	0.409^{a}	0.137	0.03
Isoleucine	0.519	0.511	0.686	0.528	0.374	0.550	0.563	0.179	0.11
Leucine	0.812^{b}	0.877^{ab}	1.309^{a}	0.926^{ab}	0.533^{b}	0.773^{b}	0.942^{ab}	0.417	0.04
Lysine	0.614^{b}	0.745^{ab}	1.102^{a}	0.697^{b}	0.444^{b}	0.674^{b}	0.775^{ab}	0.358	0.04
Phenyl-alanine	0.396	0.440	0.663	0.504	0.316	0.521	0.486	0.212	0.10
Serine	0.855	0.716	0.784	0.781	0.698	0.920	0.877	0.150	0.09
Threonine	0.886	0.835	0.904	0.864	0.736	0.928	0.987	0.180	0.46
Tyrosine	0.289	0.329	0.513	0.403	0.256	0.400	0.375	0.168	0.12
Valine	0.868^{b}	0.935^{ab}	1.234^{a}	0.865^{b}	0.485^{c}	0.687^{bc}	0.903^{ab}	0.357	0.01
Sum	10.877	10.744	13.711	11.346	8.046	11.312	12.360	3.528	0.19

[1]Protein = N*6.25, analyzed using Leco-N analyzer. [2]Amino acid determined from HPLC after hydrolysis with 6N HCl. $^{a, b, c}$ within the same row denotes significance at $p < 0.05$ by LSD multiple comparison test, n=6

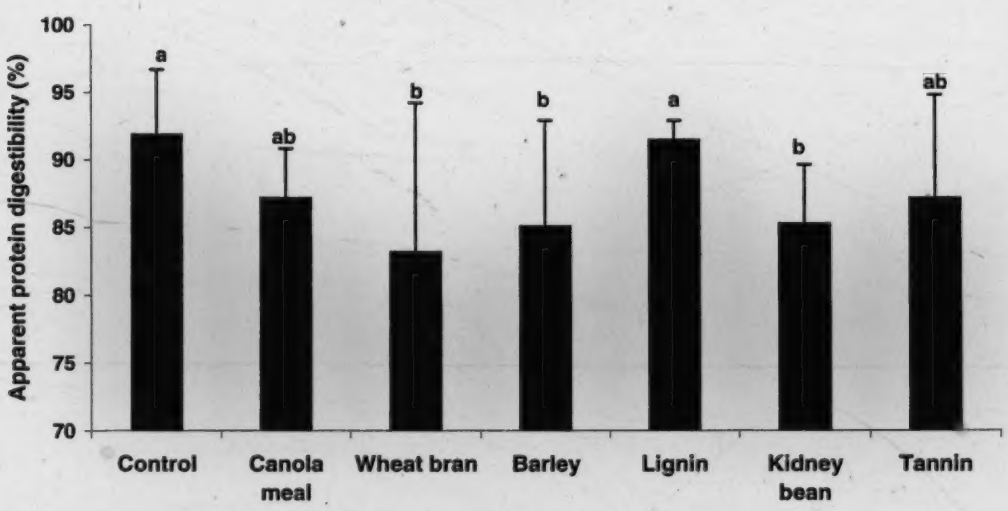
Expression of the amino acids in ileal digesta in terms of apparent digestibility showed significant differences among treatments (Table 4.3.5). Threonine and protein apparent digestibility were significantly lower for wheat bran and barley diets relative to control treatment (p=0.05 and p=0.01, respectively; see Figure 4.3.1 and Figure 4.3.2). Serine apparent digestibility was not significantly different among treatments; however, the trend (p=0.10) was similar to threonine digestibility. Apparent digestibilities were significantly different for alanine, isoleucine, valine and tyrosine; where wheat bran, barley and tannins treatments were all significantly lower than the control treatment. Though not significant (p>0.05), apparent digestibilities of arginine (p=0.06), glycine (p=0.09), histidine (p=0.08), leucine (p-0.06), lysine (p=0.07), and phenylalanine (p=0.07) showed trends towards lower values in wheat bran, barley and tannins, relative to the control.

Table 4.3.5: Apparent digestibility (%) of protein and amino acids in growing pigs fed various ANF-based test diets

Amino acids	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Protein	91.9^{a}	87.2^{ab}	83.2^{b}	85.09^{b}	91.5^{a}	85.3^{b}	87.2^{ab}	7.0	0.01
Alanine	88.46^{a}	85.53^{ab}	70.29^{c}	74.64^{bc}	88.49^{a}	80.04^{abc}	76.23^{abc}	3.72	0.04
Arginine	93.22	90.13	82.09	83.90	91.76	83.79	79.84	2.03	0.06
Aspartate	85.49	84.60	79.23	78.95	90.67	84.13	75.28	9.88	0.72
Glutamic	94.99	93.40	90.99	88.74	94.79	92.51	89.48	2.31	0.21
Glycine	79.01	75.72	62.72	67.07	76.26	61.93	52.41	4.10	0.09
Histidine	96.09	92.77	87.13	90.27	95.41	90.98	86.67	1.40	0.08
Isoleucine	94.71^{a}	91.20^{ab}	86.89^{b}	87.93^{b}	94.94^{a}	90.59^{ab}	89.34^{b}	2.01	0.02
Leucine	95.49	91.94	86.26	88.44	96.15	92.98	90.65	2.13	0.06
Lysine	95.96	91.78	86.08	89.42	96.16	92.62	90.68	1.72	0.07
Phenyl-ala	96.00	92.60	87.74	89.12	95.83	91.32	90.94	2.03	0.07
Serine	91.20	88.41	84.50	83.43	90.35	84.33	85.67	1.94	0.11
Threonine	90.64^{a}	85.53^{abc}	80.86^{c}	80.23^{c}	89.26^{ab}	83.72^{abc}	82.62^{bc}	2.36	0.05
Tyrosine	97.05^{a}	94.16^{ab}	90.71^{b}	91.04^{b}	96.54^{a}	93.14^{ab}	92.72^{ab}	1.98	0.05
Valine	93.25^{a}	87.59^{abc}	81.14^{c}	84.34^{bc}	94.95^{a}	90.99^{ab}	87.05^{abc}	2.98	0.02

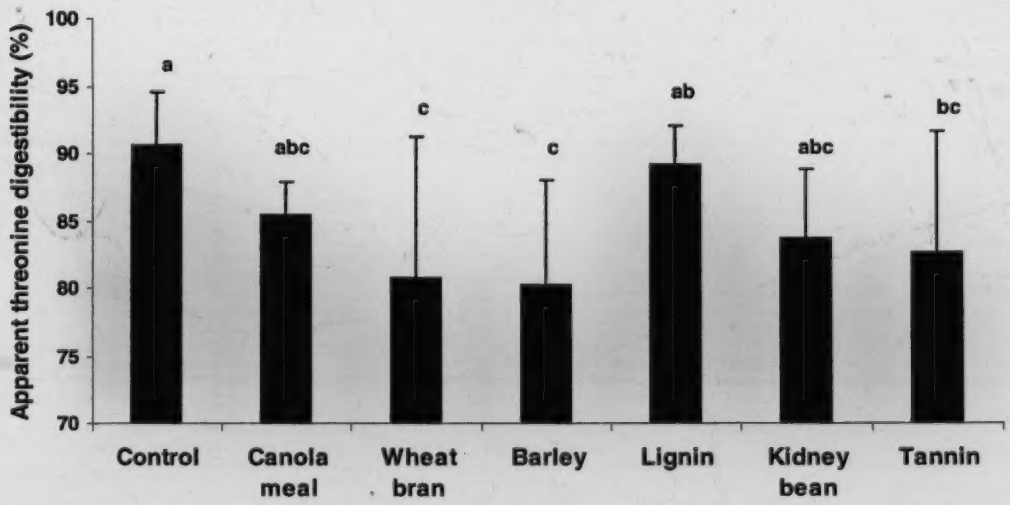
$^{a, b, c}$ within the same row denotes significance at $p < 0.05$ by LSD multiple comparison test, n=6

Figure 4.3.1: Apparent protein digestibility (%) in growing pigs fed various ANF-based dietary treatments



[a, b, c] denotes significance at $p < 0.05$ by LSD multiple comparison test, n=6

Figure 4.3.2: Apparent threonine digestibility (%) in growing pigs fed various ANF-based dietary treatments



[a, b, c] denotes significance at $p < 0.05$ by LSD multiple comparison test, n=6

4.3.2.2 Standardized Ileal Digestibilty

Standardized ileal digestibilities of protein (nitrogen) and amino acids for the casein-control diet were in agreement with results reported by other researchers (Kies et al. 1986, Furuya & Kaji 1989, Chung & Baker 1992). Though higher than apparent ileal digestibility values, the standardized threonine digestibility values were still below 90% for wheat bran, barley and tannins-based dietary treatments. There were significant differences ($p \leq 0.05$) in standardized ileal digestibilities for alanine, arginine, glycine, lysine, phenylalanine, tyrosine and the branched chain amino acids (Table 4.3.6), where wheat bran, barley and tannins-based treatments had the lowest digestibility values relative to values from the control treatment and to the other dietary treatments. On average, standardized digestibility values were 7% greater than the apparent digestibility values for all diets and their associated amino acids and protein.

Table 4.3.6: Standardized[1] digestibility (%) of protein and amino acids in growing pigs from the seven test diets

Amino acids	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Protein*	98.72	93.70	91.60	92.42	99.57	91.12	93.79	7.37	0.27
Alanine	95.24^{ab}	92.77^{abc}	76.47^{d}	80.46^{dc}	97.59^{a}	87.12^{abcd}	82.59^{bcd}	13.05	0.02
Arginine	100.00^{a}	96.36^{abc}	88.43^{dc}	89.56^{abcd}	99.70^{ab}	88.94^{bcd}	84.45^{d}	10.38	0.04
Aspartate	92.27	91.88	89.91	87.27	101.55	92.48	81.99	15.04	0.49
Glutamic acid	101.77	101.18	100.06	94.60	104.43	100.29	95.72	6.77	0.15
Glycine	85.78^{a}	81.70^{a}	69.92^{ab}	73.41^{ab}	85.06^{a}	67.68^{ab}	58.12^{b}	17.84	0.05
Histidine	102.87	98.62	93.17	95.90	103.41	96.67	90.90	8.26	0.06
Isoleucine	101.49^{ab}	98.12^{abc}	93.85^{c}	94.00^{c}	104.23^{a}	97.64^{abc}	95.81^{bc}	6.32	0.02
Leucine	102.27^{ab}	98.34^{abc}	92.78^{c}	93.84^{c}	106.48^{a}	100.82^{abc}	96.86^{bc}	8.00	0.03
Lysine	102.74^{ab}	97.47^{abc}	91.89^{c}	94.88^{bc}	105.46^{a}	99.74^{abc}	96.40^{bc}	7.92	0.04
Phenyl-alanine	102.78^{ab}	98.82^{abc}	93.99^{c}	94.10^{c}	104.31^{a}	97.07^{abc}	96.76^{bc}	6.92	0.04
Serine	97.98	96.45	93.40	90.50	98.46	90.99	92.45	6.77	0.20
Threonine	97.42	92.73	88.61	87.06	97.04	90.51	89.20	7.84	0.12
Tyrosine	103.83^{a}	100.23^{ab}	96.53^{b}	95.67^{b}	104.20^{a}	98.63^{ab}	98.15^{ab}	5.73	0.04
Valine	100.03^{ab}	93.87^{bc}	87.66^{c}	90.40^{c}	106.88^{a}	100.45^{ab}	93.74^{bc}	9.69	0.003

[1]Casein-based control diet was used to determine basal loss and digestibility. Endogenous nutrient losses (ENL) were determined from the formula by Moughan et al. (1992). ENL = N_D x (Cr_f/Cr_D), where N_D is the concentration of nutrient in the digesta, Cr_f and Cr_D are the concentration of chromic oxide in feed and digesta, respectively. Standardized digestibility (SD) was determined from the formula by Smiricky et al. (2002). SD = apparent digestibility + [(ENL/ N_D) x 100] *Protein =N*6.25 $^{a, b}$ Means within the same row with different superscripts are significantly different ($P<0.05$) by LSD multiple comparison test, n=6.

Protein and threonine standardized digestibility values were not significantly different because of the large standard deviations (see Table 4.3.6, Figure 4.3.3 and Figure 4.3.4), especially for pigs fed wheat bran-based diet.

Figure 4.3.3: Standardized protein digestibility (%) in growing pigs fed various ANF-based dietary treatments

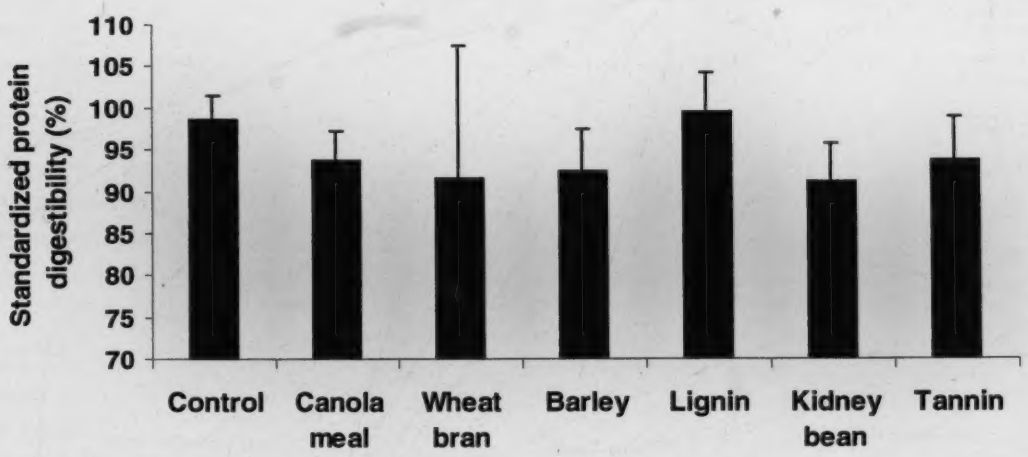
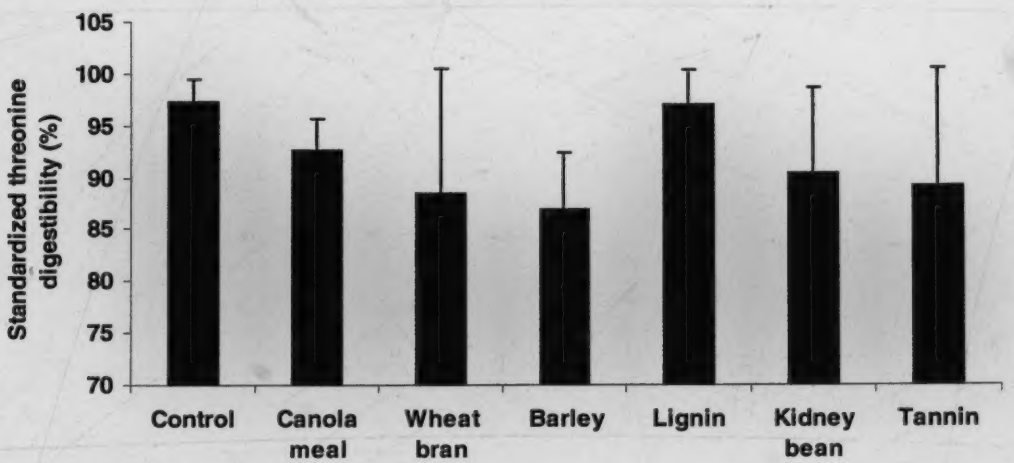


Figure 4.3.4: Standardized threonine digestibility (%) in growing pigs fed various ANF-based dietary treatments



Total ileal flow of protein, threonine and serine were highest for wheat bran and barley treatments (see Table 4.3.7). Ileal threonine flow for the control was 37.05 mg/d while wheat bran (69.1 mg/d), barley (73.5 mg/d), canola meal (56.4 mg/d) and tannins (59.1 mg/d) were all significantly (p=0.001) greater than the control and lignin (36.3 mg/d) treatments. Among dietary treatments, ileal serine flow followed a similar pattern as ileal threonine flow. In a similar trend ileal protein flow for control was 3.65 g/d, respectively, with significantly (p=0.01) higher flow for wheat bran (6.90 g/d), barley (7.53 g/d), canola meal (5.90 g/d), and lectins (5.64 g/d). The fibre contents, especially the hemicellulose component (see Table 2.3.1, Table 4.2.5) of wheat bran and barley relative to the other dietary treatments led to the greater ileal amino acid and protein losses from these two diet treatments.

Table 4.3.7: Total flow of protein (g/d) and amino acid (mg/d) at the distal ileum in growing pigs after ingestion of various ANF-based diets

Amino acids	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Protein*	3.65^{d}	5.90^{abc}	6.90^{ab}	7.53^{a}	4.72^{cd}	5.64^{abc}	5.51^{bcd}	1.89	0.01
Alanine	26.51^{b}	41.10^{ab}	71.41^{a}	64.31^{a}	25.33^{b}	45.88^{ab}	46.45^{ab}	28.89	0.03
Arginine	19.12	33.67	52.59	48.27	22.00	45.70	46.39	25.33	0.09
Aspartate	86.77	100.23	117.94	127.05	52.42	92.78	137.24	101.43	0.83
Glutamic acid	86.79	119.10	149.86	206.26	84.98	126.85	165.94	97.78	0.28
Glycine	30.36	55.45	64.31	66.89	32.46	57.12	59.00	27.18	0.10
Histidine	9.51	17.98	29.37	23.80	10.67	21.44	25.53	13.90	0.09
Isoleucine	21.08^{b}	34.78^{ab}	48.32^{a}	48.69^{a}	19.45^{b}	36.91^{ab}	36.56^{ab}	19.47	0.03
Leucine	34.15	60.09	95.23	88.01	27.96	52.17	62.49	45.77	0.13
Lysine	25.50	50.69	79.33	65.68	23.31	45.75	50.34	36.83	0.12
Phenyl-alanine	16.49^{b}	30.15^{ab}	46.93^{a}	46.08^{a}	16.49^{b}	35.23^{ab}	31.77^{ab}	21.90	0.05
Serine	34.80	48.43	60.16	69.84	36.76	61.47	57.14	23.77	0.07
Threonine	35.74^{b}	56.33^{ab}	69.04^{a}	76.63^{a}	39.51^{b}	61.62^{ab}	62.27^{ab}	25.60	0.04
Tyrosine	12.10	22.75	34.81	36.84	13.57	27.39	25.10	16.70	0.06
Valine	35.28^{b}	63.85^{ab}	91.33^{a}	82.42^{a}	25.43^{b}	46.22^{b}	60.17^{ab}	39.59	0.02

* Protein =N*6.25 $^{a, b, c, d}$ Means within the same row with different superscripts are significantly different (P<0.05) by LSD multiple comparison test, n=6.

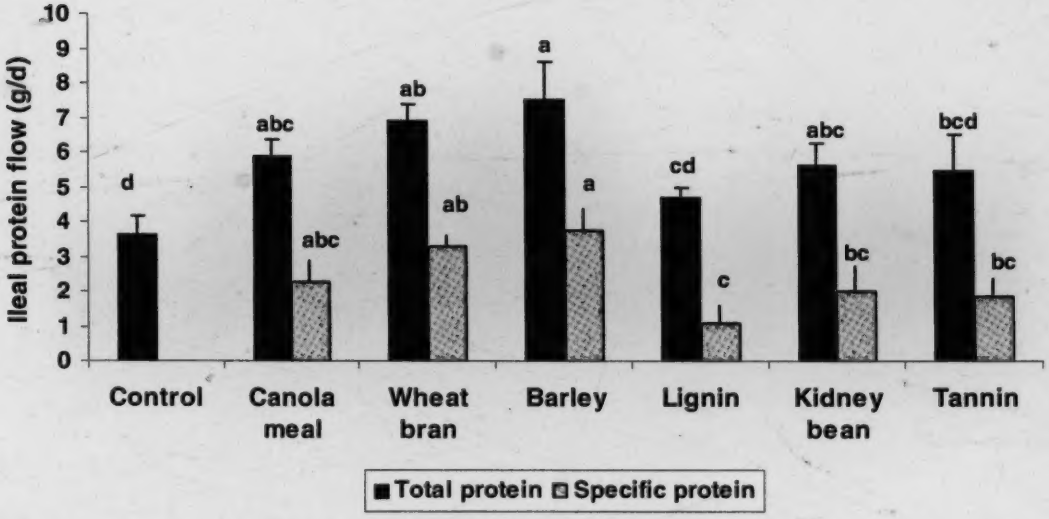
After correcting for the basal loss of protein and amino acids there was a significant difference (p=0.03) in protein loss among the six ANF dietary treatments; barley and wheat bran treatment had significantly higher values compared to lignin treatment. Overall, the greatest loss occurred in the barley treatment. Once corrected for basal loss, amino acid losses among dietary treatments were not significantly different, except for valine.

Table 4.3.8: Ingredient specific flow of protein (g/d) and amino acid (mg/d) at the terminal ileum in growing pigs after ingestion of various ANF-based diets, corrected for basal[1] flow

Amino acids	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Protein*	N/A	2.25^{abc}	3.30^{ab}	3.74^{a}	1.07^{c}	1.99^{bc}	1.86^{bc}	1.22	0.03
Alanine	N/A	14.59	45.67	39.07	-1.18	19.37	19.94	12.56	0.06
Arginine	N/A	14.55	33.80	30.62	2.88	26.58	27.27	13.78	0.25
Aspartate	N/A	13.45	32.55	38.03	-34.35	6.00	50.46	107.78	0.71
Glutamic acid	N/A	32.31	65.00	126.74	-1.82	40.06	79.15	77.21	0.21
Glycine	N/A	25.09	34.72	35.19	2.10	26.76	28.65	22.15	0.16
Histidine	N/A	8.47	20.21	14.44	1.16	11.93	16.02	5.95	0.26
Isoleucine	N/A	13.70	27.84	29.38	-1.62	15.84	15.48	8.01	0.07
Leucine	N/A	25.93	62.34	49.75	-6.20	18.01	28.33	17.10	0.13
Lysine	N/A	25.19	55.04	36.43	-2.19	20.25	24.84	11.22	0.17
Phenyl-alanine	N/A	13.66	31.00	31.36	0.00	18.73	15.28	7.22	0.13
Serine	N/A	13.63	25.97	36.49	1.96	26.67	22.34	13.20	0.15
Threonine	N/A	20.59	33.95	42.55	3.77	25.88	26.53	13.61	0.17
Tyrosine	N/A	10.65	23.25	25.17	1.47	15.29	13.00	6.35	0.14
Valine	N/A	28.56^{abc}	57.07^{a}	52.62^{ab}	-9.85^{c}	10.93^{bc}	24.89^{abc}	13.84	0.03

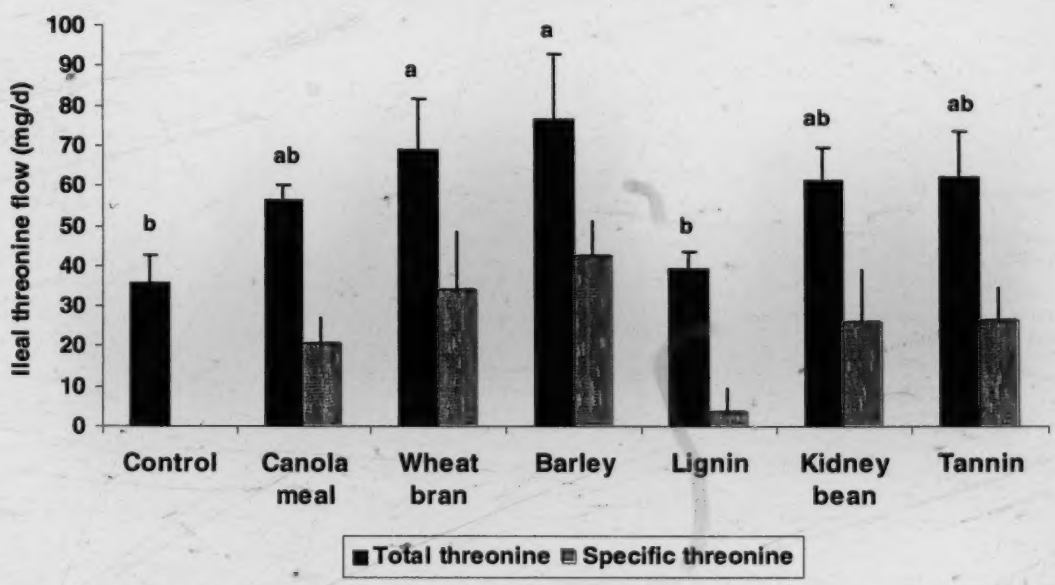
[1] basal loss is calculated as ileal amino acid and N flow from the semi-synthetic casein-based control diet. * Protein =N*6.25 $^{a, b}$ Means within the same row with different superscripts are significantly different (P<0.05) by LSD multiple comparison test, n=6.

Figure 4.3.5: Total and ingredient-specific ileal protein flow (g/d) in growing pigs fed various ANF-based dietary treatments



[a, b, c] denotes significance at $p < 0.05$ by LSD multiple comparison test, n=6

Figure 4.3.6: Total and ingredient-specific ileal threonine flow (mg/d) in growing pigs fed various ANF-based dietary treatments



[a, b, c] denotes significance at $p < 0.05$ by LSD multiple comparison test, n=6

4.3.2.3 Fecal Digestibility

Table 4.2.5 shows the fibre content of the experimental diets. Barley-, wheat bran- and canola meal-based diets had greater NDF and ADF compared to the control diet. NDF and ADF contents for lignin, kidney beans and tannins-based diets were not numerically much different from the control diet. The difference between the NDF and ADF values of a feedstuff gives the hemicellulose content of the feedstuff. Since the NDF and ADF content of barley, wheat bran and, canola meal-based diets were greater than the control diet, it followed that the hemicellulose contents of these diets would be greater than the control, as is seen in Table 4.2.5. Because of its relatively small quantity in feedstuffs, lignin is one of the more challenging fibre components to measure in feedstuffs. In this experiment the overall fibre contents of the test diets were relatively low; as such, the lignin content is even lower. Barley, wheat bran, canola meal and lignin diets had similar lignin contents, which were significantly greater than the control diet. Kidney beans and tannins-based diets had lower lignin contents than the other diets, except for the control diet, which had the lowest content. In fact, because the lignin content of the control diet was so low, analysis using the fibre-bag technique was calculated as negative. Cellulose contents were similar for barley, wheat bran, canola meal, and control diets. Lignin, kidney beans and tannins-based diets had similar cellulose content, which was significantly lower than the control diet.

Fecal digestibility was calculated from the fibre compositions of the dietary treatments (Table 4.3.9) and fibre compositions of the fecal samples taken from animals fed the dietary treatments. Fecal digestibility for all fibre components was lowest for the lignin treatment (Table 4.3.10), followed by tannins, then wheat bran and kidney beans treatments.

Table 4.3.9: Fibre composition (g/kg DM) of fecal samples from experimental animals fed ANF-based dietary treatments

Component	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans)	Tannins
NDF	55.85	54.32	52.84	51.66	41.76	51.14	48.29
NDR	70.48	62.53	57.55	59.30	49.28	64.31	57.38
ADF	35.56	33.76	22.57	28.49	27.94	31.29	30.94
HC	20.27	20.51	30.22	23.12	13.84	19.80	17.31
Cellulose	29.46	15.84	16.01	21.52	20.75	22.08	20.51
TC	49.74	36.35	46.23	44.64	34.60	41.87	37.82
Lignin	5.11	16.96	5.58	6.01	6.21	8.25	9.45

NDF = neutral detergent fibre, NDR = neutral detergent residue, ADF= acid detergent fibre, HC= hemicellulose, TC= total cellulose, i.e., HC and cellulose

Table 4.3.10: Fecal digestibility (%) of fibre components and nutrients in growing pigs fed various ANF-based test diets[1]

Fecal Digestibility	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
NDF	90.68^{abc}	91.76^{ab}	88.58^{bc}	93.35^{a}	67.46^{e}	87.67^{c}	82.13^{d}	9.13	<0.0001
NDR	90.32^{ab}	91.70^{ab}	88.58^{bc}	92.95^{a}	70.10^{e}	86.68^{c}	82.82^{d}	8.21	<0.0001
ADF	71.27^{b}	81.53^{a}	78.25^{ab}	81.55^{a}	33.43^{d}	50.81^{c}	43.73^{c}	21.47	<0.0001
HC	95.74^{ab}	95.69^{ab}	91.58^{c}	96.23^{a}	88.97^{d}	94.35^{b}	91.96^{c}	4.48	<0.0001
Cellulose	90.88^{ab}	91.81^{a}	81.83^{cd}	84.51^{bc}	21.83^{f}	76.18^{d}	68.78^{e}	23.77	<0.0001
Lignin	140.12^{ab}	71.89^{b}	75.74^{b}	85.73^{ab}	65.96^{b}	ND	191.38^{a}	387.15	<0.0001
DM	97.07^{a}	94.23^{bc}	95.64^{d}	93.21^{cd}	92.95^{d}	96.51^{a}	94.73^{b}	2.15	<0.0001
Ash	ND	9.83	ND	ND	ND	ND	ND	55.49	<0.0001
Protein	97.3^{a}	93.5^{b}	93.3^{b}	93.7^{b}	92.3^{b}	95.5^{a}	93.1^{b}	2.20	0.0001
Energy	96.2^{a}	90.8^{bc}	80.9^{d}	86.9^{c}	80.5^{d}	94.7^{ab}	90.6^{bc}	6.74	<0.0001
DE (kcal/g)	3686.3	3657.5	3292.3	3855.5	3195.4	3661.8	3564.6		

[1]Calculated using DM fibre values from diet and fecal samples. $^{a, b, c}$ within the same row denotes significance at $p< 0.05$ by LSD multiple comparison test, n=6. Chromic oxide was used as the digestibility marker. NDF = neutral detergent fibre, NDR = neutral detergent residue, ADF= acid detergent fibre, HC= hemicellulose, TC= total cellulose, i.e., HC and cellulose. DE= digestibility energy. ND= not detected.

4.3.3 Gut Morphology, Organ and Tissue Weights in Growing Pigs Fed Various ANF-based Diets

4.3.3.1 Intestinal Weights and Length

Overall small intestinal weight and length were not significantly different among dietary treatments. However, the overall large intestinal weight of the lignin treatment group was significantly ($p=0.01$, see Table 4.3.11) greater than all other dietary treatments. Total small intestinal weight-per-body weight was significantly greater for canola meal treatment ($p=0.05$) relative to control, while the large intestinal weight-per-body weight was significantly ($p<0.01$) higher for lignin treatment. The large intestinal weight-per-length was also highest for lignin treatment ($p=0.01$).

Table 4.3.11: Intestinal lengths and weights of growing pigs fed various ANF-based diets

Parameter	Units	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Small intestine										
Weight	g	423.42	509.32	523.13	428.22	453.7	557.3	469.76	184.06	0.08
Wt/ BW	g/kg	26.25^{c}	44.37^{a}	39.22^{ab}	28.64^{bc}	30.11^{bc}	35.87^{abc}	29.24^{bc}	10.86	0.05
Length	cm	1265.8	1383.0	1268.7	1360.7	1252.2	1470.2	1330.2	258.2	0.50
Wt/ Length	g/cm	0.33	0.37	0.41	0.31	0.36	0.38	0.35	0.16	0.39
Large intestine										
Weight	g	165.33^{b}	180.4^{b}	203.74^{a}	161.94^{b}	262.35^{a}	184.55^{b}	157.8^{b}	48.38	0.01
Wt/ BW	g/kg	10.30^{c}	12.28^{bc}	14.52^{ab}	10.85^{c}	17.61^{a}	11.93^{bc}	10.01^{c}	3.17	0.001
Length	cm	248.7	243.7	235.2	252.4	239.7	277.2	226.7	61.06	0.58
Wt/ Length	g/cm	0.66^{b}	0.74^{b}	0.87^{b}	0.64^{b}	1.09^{a}	0.67^{b}	0.70^{b}	0.22	0.01

$^{a, b, c}$ within the same row denotes significance at $p < 0.05$ by LSD multiple comparison test, n=6. Wt = weight, BW = body weight

4.3.3.2 Intestinal Sections

Ileum mucosal weight/ length was significantly ($p<0.001$) greater for wheat bran treatment compared to all other dietary treatments (Table 4.3.12). Colon mucosal weight/ length was significantly greater for lignin treatment compared to all other dietary treatments.

Table 4.3.12: Effects of ANF on intestinal sections: weights/length (g/cm) in growing pigs

Parameter: wt./length	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
				Duodenum					
Sample[1]	0.36	0.42	0.40	0.38	0.36	0.35	0.44	0.66	0.41
Mucosal	0.21	0.24	0.23	0.21	0.20	0.24	0.24	0.15	0.65
Muscularis	0.12	0.18	0.17	0.18	0.16	0.11	0.20	1.11	0.40
				Mid Jejunum					
Sample	0.34	0.36	0.34	0.30	0.36	0.42	0.32	0.57	0.37
Mucosal	0.28	0.28	0.27	0.23	0.29	0.33	0.24	0.06	0.15
Muscularis	0.07	0.08	0.07	0.07	0.08	0.09	0.08	0.13	0.43
				Distal Jejunum					
Sample	0.30	0.41	0.28	0.27	0.30	0.33	0.29	0.98	0.28
Mucosal	0.21	0.32	0.22	0.20	0.22	0.24	0.22	0.09	0.13
Muscularis	0.09	0.08	0.06	0.07	0.08	0.09	0.07	0.03	0.42
				Ileum					
Sample	0.48^{b}	0.74^{b}	1.36^{a}	0.50^{b}	0.45^{b}	0.55^{b}	0.66^{b}	0.56	0.001
Mucosal	0.23^{b}	0.38^{b}	0.51^{a}	0.24^{b}	0.24^{b}	0.29^{b}	0.30^{b}	0.18	0.001
Muscularis	0.25	0.36	0.85	0.26	0.21	0.26	0.37	1.45	0.16
				Mid Colon					
Sample	0.63^{bc}	0.68^{bc}	0.77^{b}	0.52^{bc}	1.07^{a}	0.63^{bc}	0.47^{c}	0.23	0.0004
Mucosal	0.17^{b}	0.18^{b}	0.21^{b}	0.17^{b}	0.40^{a}	0.16^{b}	0.13^{b}	0.10	0.001
Muscularis	0.46	0.48	0.57	0.35	0.67	0.47	0.34	0.36	0.26

$^{a, b, c}$ within the same row denotes significance at $p< 0.05$ by LSD multiple comparison test, n=6

[1]Sample represent the fraction of each segement of the small intestine which was use to represent the intact intestine, i.e, musocosal and muscularis.

Digital photographs were taken of the ileums of pigs fed all the treatments. In particular, note the obvious structural differences between two representative ileum samples from the wheat bran treatment and controls (Picture 4.3.1). Unlike the other dietary treatments, pigs on wheat bran-based dietary treatment had more muscular ileum than pigs on the casein-based treatment. Relative to barley and canola meal, wheat bran is a very bulky feedstuff. The bulkiness of wheat bran may play a role in why pigs on wheat bran-based diets had such muscular ileums. Thus, the bulkiness of the wheat bran may slow along the movement of digesta; hence, the animal had to work harder in moving digesta along the small intestine. The other treatments were visually similar to controls as supported by data in Table 4.3.12. Interestingly, as noted in Table 4.3.12, there also appeared to be more mucosa and thicker muscularis in the colons of animals fed the lignin-based treatment compared to the other treatments. Because of difficulties in getting good pictures from the colon samples, these images are not shown.

Photograph 4.3.1: Structure of the ileum of a typical pig fed a wheat bran-based diet compared to a pig fed a casein-based control diet

Ileum of a Wheat Bran Fed Pig vs. a Control Pig
WHEAT BRAN (B)
REP #6 OCT 22/01
OCT 26/01
Wheat Bran compared to Control. Final pig weight is 15.84 kg and 15.78 kg, respectively. Both pigs are from replication 6 of 6.

4.3.3.3 Organs

Organ weights (both absolute and corrected for body weight) were similar among all dietary treatments. Additionally, there was no atrophy or discolouration noted among the organs from the animals fed the various experimental diets.

Table 4.3.13: Comparisons of organ weights of growing pigs fed various ANF-based diets (g)

Organ	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Liver	426.70	470.23	414.52	437.08	446.18	387.05	400.35	82.57	0.74
Spleen	41.50	43.92	42.37	35.81	44.97	33.35	45.30	14.98	0.34
Kidney	88.13	81.15	72.94	74.26	80.73	83.43	83.38	14.54	0.21
Stomach	121.43	138.10	144.82	148.16	133.85	104.43	131.05	60.87	0.28

Table 4.3.14: Specific organ weights of growing pigs fed various ANF-based diets (g/kg BW[1])

Organ	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Liver	26.68	23.91	30.36	29.93	29.40	25.10	25.00	5.49	0.21
Spleen	2.60	3.26	3.10	2.36	2.98	2.16	2.88	0.98	0.44
Kidney	5.52	5.71	5.44	4.97	5.32	5.42	5.19	0.84	0.84
Stomach	7.55	12.79	10.74	9.99	8.98	6.76	8.38	3.38	0.17

[1]BW = live pig body weight at necropsy

4.3.4 Plasma Amino Acids

Threonine, serine and proline, the three main amino acids found in mucin were not found to be significantly different among treatments. Plasma cysteine levels were significantly higher for canola meal (28 μmol/L) and wheat bran (22 μmol/L) relative to the other dietary treatments. Reflective of glycine addition to the diets, plasma glycine concentrations were highest in barley-fed pigs relative to all other dietary treatments. Canola meal, barley and wheat bran pigs had significantly ($p=0.016$) higher plasma histidine levels (55, 53, and 49 μmol/L, respectively) relative to control treated pigs (38 μmol/L).

Figure 4.3.7: Plasma threonine concentration (μmol/L) in growing pigs receiving various ANF-based diets

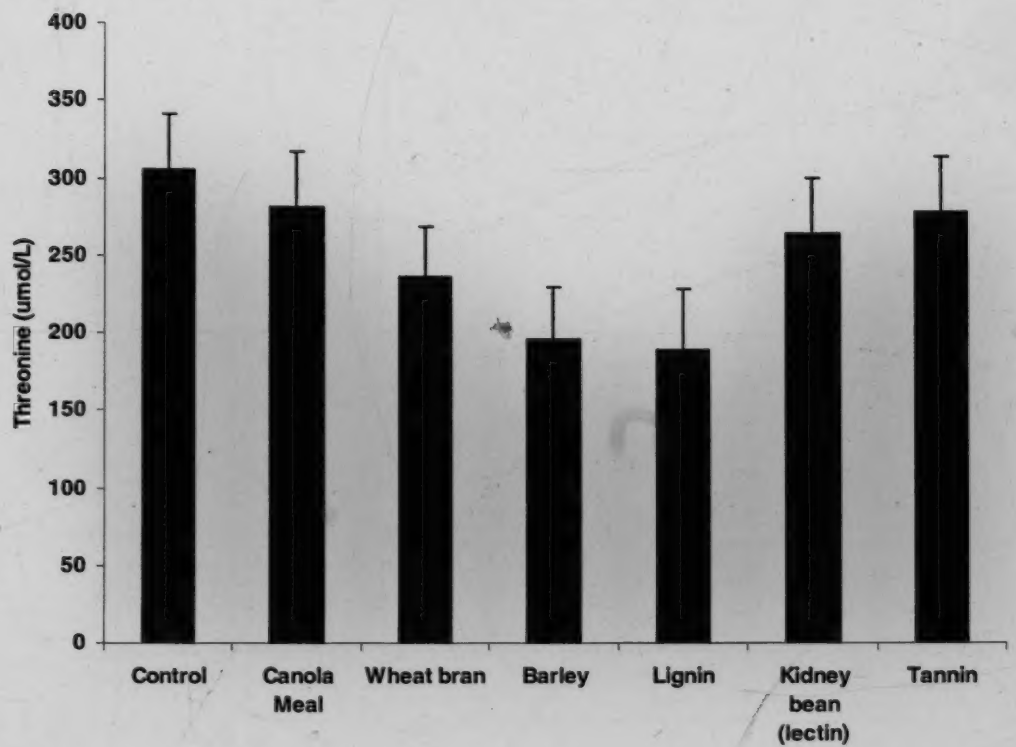


Table 4.3.15: Plasma amino acid concentration (μmol/L) in growing pigs fed various ANF test diets, as determined by HPLC analysis

Amino acids	Control	Canola Meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Ala	871.52	903.54	851.10	776.02	764.28	931.11	824.31	280.42	0.95
Arg	85.70	102.62	114.16	125.43	94.47	82.70	80.43	36.18	0.18
Asp	165.36	165.07	157.92	106.77	214.03	162.66	175.81	93.50	0.68
Cys	4.81^{b}	27.80^{a}	22.04^{a}	7.66^{b}	5.53^{b}	6.90^{b}	6.53^{b}	11.59	<0.0001
Gln	578.48	640.85	550.05	600.89	547.11	638.93	639.25	212.01	0.97
Glu	296.41	392.07	474.51	367.12	355.59	320.46	286.47	134.06	0.16
Gly	518.64^{b}	568.66^{ab}	613.88^{ab}	687.95^{a}	475.69^{b}	602.52^{ab}	503.62^{b}	130.56	0.05
His	38.49^{bc}	55.45^{a}	49.34^{ab}	53.53^{a}	30.01^{c}	41.99^{abc}	38.55^{bc}	14.48	0.02
Hyp	126.63	123.29	104.68	99.36	75.59	132.92	115.83	34.81	0.09
Ile	178.51	176.10	190.54	160.83	165.74	167.32	146.03	53.97	0.87
Leu	180.32	191.70	183.50	177.81	161.41	181.13	159.34	53.07	0.95
Lys	88.70	118.82	168.64	96.68	118.67	129.16	74.12	86.01	0.55
Met	30.18	33.25	27.34	24.02	23.97	27.88	30.43	11.69	0.82
Phe	59.66	68.87	61.80	75.17	50.75	72.27	68.75	17.05	0.20
Pro	502.44	397.69	503.07	427.59	478.39	446.56	426.92	168.67	0.92
Ser	163.37	159.32	160.36	193.61	152.65	183.85	155.08	41.38	0.51
Tau	117.41	150.69	136.05	172.89	91.46	116.60	118.25	48.14	0.07
Thr	305.08	281.21	235.41	195.51	188.71	263.43	277.81	90.99	0.19
Tyr	133.20	122.43	112.32	93.45	99.48	114.81	131.27	44.12	0.66
Val	346.09	382.52	361.03	312.32	278.26	325.90	319.06	97.59	0.67
Sum	5291.01	5561.95	5577.75	5254.62	4871.78	5449.12	5077.85	1073.42	0.93

$^{a, b, c}$ within the same row denotes significance at $p< 0.05$ by LSD multiple comparison test, n=6

4.3.5 Animal Performance

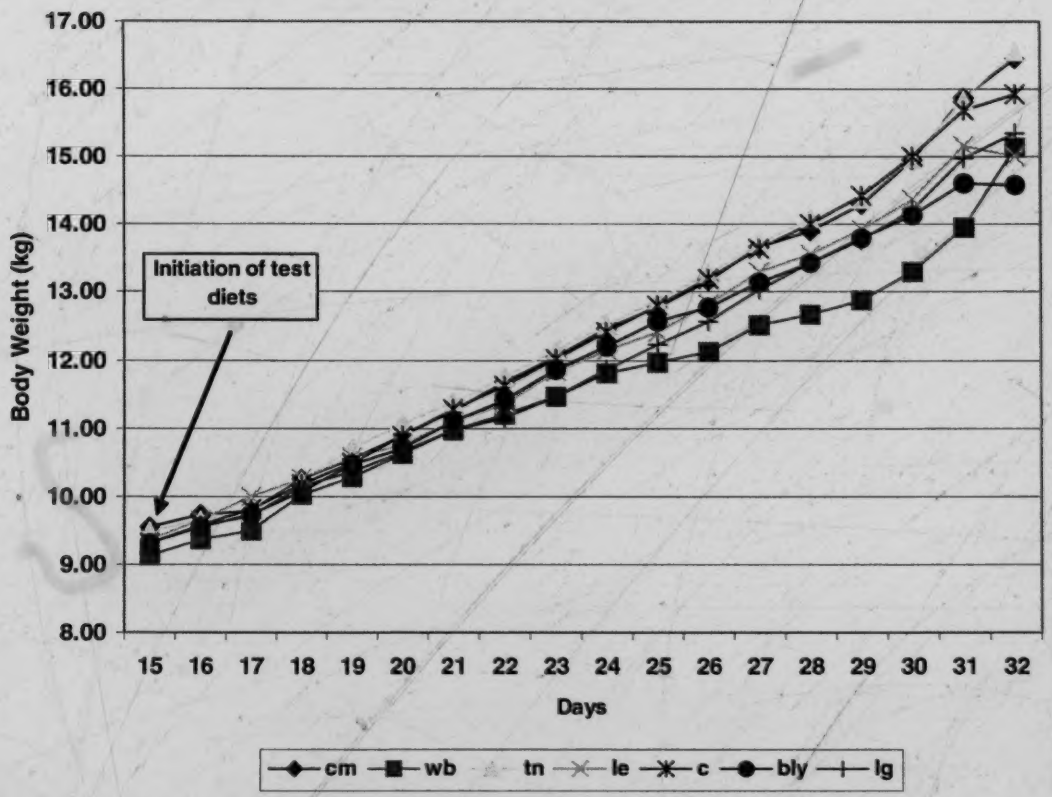
During the course of the study, pigs were healthy and active, with pigs fed at 5% of daily BW. For all pigs, the average initial weight at the start of experimental treatments was 9.45 kg (SD= 0.71), the average final weight was 15.57 kg (SD=0.77), and the overall average weight during treatment period was 12.06 kg (SD= 0.95). As seen in Figure 4.3.8, all pigs grew during the study period. Though initial weights were not different among treatments (p=0.99), there were significant differences in final weights among treatments (p<0.01, Table 4.3.16). All animals experienced similar body weight gain during the first week (d 15 to d 24) on the dietary treatments; however, during the second week (d 25 to d 33) there were more noticeable differences in body weight gain, with wheat bran treated pigs growing the least (Figure 4.3.8). Over the course of the study, average daily feed intake was 0.57 kg (SD= 0.06), and average daily gain was 0.37 kg (SD= 0.07). As seen below in Table 4.3.16, overall average daily gain was significantly different (p= 0.05). Most importantly, average daily gains (ADG) for pigs fed wheat bran supplemented diets were lower than for pigs fed the control, canola meal and tannins supplemented diets. Overall, feed conversion ratio (FCR), revealed no significant differences among treatments.

Table 4.3.16: Weight changes, average feed intake and feed conversion ratios in growing pigs fed various ANF test diets

Parameter	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Initial Wt (kg)	9.31	9.64	9.26	9.36	9.34	9.44	9.58	0.14	0.56
Final Wt (kg)	16.00^{a}	16.23^{a}	14.81^{b}	15.24^{ab}	15.22^{ab}	15.36^{ab}	16.10^{a}	0.77	0.004
ADI (kg)	0.59	0.58	0.56	0.57	0.58	0.59	0.59	0.06	0.52
ADG (kg)	0.41^{a}	0.41^{a}	0.31^{b}	0.34^{ab}	0.36^{ab}	0.37^{ab}	0.41^{a}	0.07	0.05
FCR	0.67	0.66	0.53	0.57	0.59	0.59	0.66	0.13	0.34

ADI = average daily intake; kg, ADG = average daily gain; kg, FCR = feed conversion ratio (gain: intake). Mean is the average weight all pigs during the treatment period. $^{a, b, c}$ within the same row denotes significance at p< 0.05 by LSD multiple comparison test, n=6

Figure 4.3.8: Growth performance of growing pigs receiving various ANF



cm = canola meal, wb = wheat bran, tn = tannins, le = kidney beans lectins, c = control, bly = barley, lg = lignin

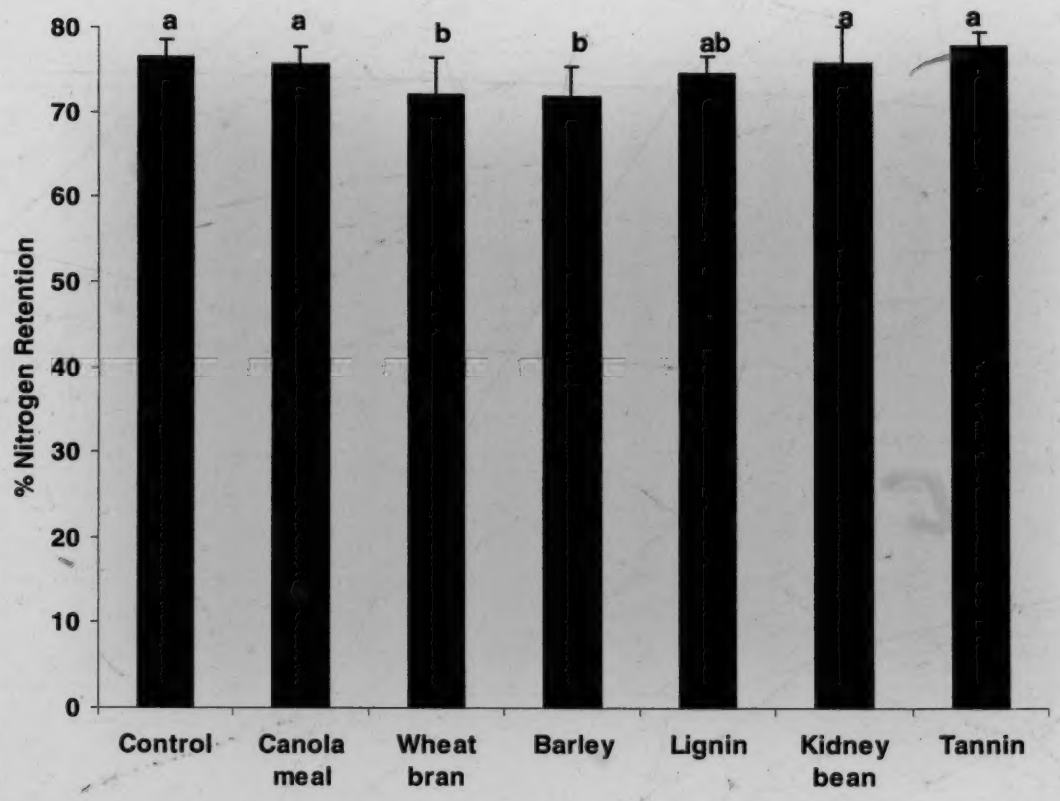
Following a week (7 days) of adaptation to the test diets, nitrogen balance was then conducted for 5 days. During the course of the five days, nitrogen intake and excretion were similar among treatment groups (p = 0.28 and p=0.32, respectively). However, there was a trend (p=0.06) towards lower N retention (g/d) for wheat bran and barley treated pigs. In addition, these treatment groups had significantly lower nitrogen retention (p=0.01).

Table 4.3.17: Nitrogen balance in growing pigs fed various ANF-based diets

Nitrogen parameters	Units	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins	Std dev	p
Intake	g N/d	22.50	22.49	19.47	21.83	21.88	22.34	22.35	2.52	0.28
Excretion	g N/d	5.31	5.49	5.50	6.12	5.55	5.41	4.91	0.85	0.32
Retention	g N/d	17.19	17.00	13.97	15.71	16.33	16.93	17.43	2.25	0.06
Retention	%	76.44^{a}	75.57^{a}	71.93^{b}	71.84^{b}	74.64^{ab}	75.87^{a}	77.93^{a}	3.67	0.01
Retention/ BW	gN/kg/d	1.01^{a}	1.06^{a}	0.88^{b}	1.05^{a}	1.08^{a}	1.05^{a}	1.04^{a}	0.12	0.03

$^{a, b, c}$ within the same row denotes significance at $p < 0.05$ by LSD multiple comparison test, n=6. N-intake determined by feed intake * N content of feed as determined by Leco-Nitrogen analysis of the diets. N-excretion determined from N loss in feces and through urine, N in feces and urine were analyzed by Kjeldahl -N

Figure 4.3.9: Nitrogen retention (%) in growing pigs receiving various ANF-based diets with identical threonine intakes



[a, b] denotes significance at $p < 0.05$ by LSD multiple comparison test, n= 6

4.3.6 Summary

Table 4.3.18 summarizes the main results from this experiment relative to the control diet; which is designated as "zero". The responses to test parameters are presented using plus or minus symbols to indicate values greater or less than control, respectively. Within a row, multiple symbols are different from one another (ie "++" is significantly greater than "+" is significantly greater than 0, etc.). This approach was chosen to simplify the comparison of the large number of factors, with different units and clarify thedirection and degree of difference between diets.
In summary, feed ingredients that caused higher mucin flow (e.g. barley) corresponded to lower amino acid/protein digestibility values, greater flow of amino acid/protein at the terminal ileum, reduced plasma amino acid concentration and lower nitrogen retention. Also, complete feeds with the various ANFs affected mucin secretion but had little effect on intestinal mucosal weights other than in the ileum. Lignin dietary treatment exhibited most of its effect in the large intestine with no effect on the small intestine, which was quite different from all other dietary treatments used in this experiment.

Table 4.3.18: Summary of growing pigs responses to various ANFs: effect on mucin output, threonine digestibility, intestinal mucosal secretion and animal performance

Parameter	Control	Canola meal	Wheat bran	Barley	Lignin	Kidney beans	Tannins
Mucin output							
CrdM flow	0	+	++	+++	+++	+	0
Mucin	0	+	++	+++	+	+	+
Threonine ileal digestibility and ileal flow							
Apparent	0	-	---	---	-	--	--
Standard	0	-	--	---	0	-	--
Total flow	0	+	++	++	0	+	+
Protein ileal digestibility and ileal flow							
Apparent	0	-	--	--	0	--	-
Standard	0	-	-	-	0	-	-
Total flow	0	++	+++	++++	+	++	++
Intestinal mucosal weight							
Ileum	0	+	++	0	0	+	+
Distal-jej	0	+	0	0	0	0	0
Mid-jej	0	0	0	0	0	+	0
mid-colon	0	0	0	0	++	0	0
Plasma threonine and Animal performance							
Plasma	0	0	0	0	0	0	0
N-Retention	0	0	--	-	-	0	0
FCR	0	0	0	0	0	0	0

Within a row, multiple symbols are different from one another (ie "++" is significantly greater than "+" is significantly greater than 0, etc.).

4.4 Discussion

There are various anti-nutritional factors (ANF) in pig feedstuffs that may impact mucosal secretion in the animal (Pusztai 1988, Jansman 1993, Satchithandam et al. 1990). Mucin, the primary protein of mucosal secretions, is rich in threonine (Lien et al. 1997, Allen et al. 1981), and mucin is not recycled for use by the animal (Hoskins 1984, van der Schoor 2002). Additionally, it is known that some anti-nutritional factors in pig feedstuffs are more potent than others in stimulating mucosal secretion (Pusztai 1988, Jansman 1993, Liener 1990). Thus, we hypothesized that the more potent the ANF in stimulating mucosal secretion, the greater the mucin output, and consequently the more threonine loss from the animal.

The main objective of this experiment was to determine which of six ingredients: --three ANF ingredients (tannins, lignin and lectins in red kidney beans) and three high fibre pig feedstuffs (barley, canola meal and wheat bran) -- would generate the greatest mucin output from the small intestine of growing pigs. The main results of this experiment clearly showed that of the seven dietary treatments used in this study, 15% barley in the diet had the greatest impact on ileal mucin output (Table 4.3.1 and Table 4.3.2) and ileal threonine loss (Table 4.3.7 and Table 4.3.8) from the small intestine of the growing pig. To further support our findings for mucin output and threonine loss relative to the animal's diet, there were several other responses measured in this experiment. This discussion will attempt to connect all the measured parameters as they relate to mucin output and the dietary treatments utilized for this study.

Mucin versus Crude Mucin

The recovery of mucin from the small intestine, and the measurement of mucin flow along the gastrointestinal tract, was intended to provide insights into the effect of diet components on gastrointestinal epithelium physiology (Lien et al. 1997, Montagne et al. 2000). But, despite the interesting information that could be obtained from such measurements, information on the recovery of mucin in digesta and on daily mucin output is scarce, probably due to the difficulty in purifying and assaying mucin. Few researchers (Fuller & Cadenhead 1991, Lien et al. 1997, Montagne et al. 2000) have attempted to quantitatively measure mucin output from ileal digesta of animals, by measuring 'crude mucin' based on a precipitation technique or mucin based on galactosamine and glucosamine as markers.

The recovery of crude mucin (soluble mucin) is not a complete representation of mucin from ileal digesta, because the isolation of mucin from digesta is compounded by the presence of undigested dietary materials and incomplete solubility of mucin (Clamp & Gough 1991, Allen 1981, Lien 1995). Crude mucin, the water soluble-ethanol precipitable fraction of ileal digesta (Lien et al. 1997), has been noted to contain a large proportion of nonmucus protein. Removal of these contaminants prove to be a long and laborious procedure and not always feasible for large sample sizes (Clamp & Gough 1991, Allen 1981, Lien et al. 1995). Therefore, because it is impossible to obtain complete, uncontaminated isolation of mucin from ileal digesta, we determined both crude mucin output and mucin output using markers. Mucin output was estimated by the use of amino-carbohydrate (hexosamine) markers, namely N-acetyl-galactosamine (GalNAc) and N-acetyl-glucosamine (GlcNAc) (Mariscal-Landin et al. 1995, Lien et al 1995, Montagne et al. 2000).

Several studies (Lien et al. 1995, 1997, Allen 1981, Montagne et al. 2000a & b) have shown that mucin is the primary source of endogenous carbohydrates in ileal effluent and digesta from humans and animals, where galactose, fucose and hexosamines (GalNAc and GlcNAc) accounted for 77-90% of the total carbohydrates of mucin. The hexosamines have been used to estimate the ileal mucin output in pigs, humans and calves (Fuller & Cadenhead 1991, Mariscal-Landin et al. 1995, Lien et al. 1995, 1997, Montagne et al. 2000). N-acetyl-galactosamine has been accepted as well-suited for the determination of mucin flow because of its limited occurrence in diets and other endogenous sources. Glycoconjugates from bacteria or epithelial cells have been shown to be very low and limited sources of GalNAc in ileal digesta (Montagne et al. 2000). However, the use of GlcNAc as a marker for mucin is more questionable due to its contribution from other sources such as proteoglycans (Lien et al. 1995, Montagne et al. 2000a & b).

Unfortunately, it is not possible to use only GalNAc to determine mucin output because mucin at the distal ileum is a representation of mucin secretion along the entire gastrointestinal tract. Because mucin is not degraded until it reaches the large intestine (Hoskins et al. 1984, van der Schoor et al. 2002), any mucin collected from thedistal ileum will be representative of both gastric and small intestinal mucin. In pigs, the mucin carbohydrate composition changes along the gastrointestinal tract, where gastric mucin contains approximately 30% glucosamine and 13% galactosamine, while intestinal mucin contain approximately 20% and 40%, respectively (Lien et al. 1995, 1997, Scawen & Allen 1977, Mantle & Allen 1981). Thus, the accuracy in determining the carbohydrate composition of mucin from crude mucin and ileal digesta will determine the reliability of the estimates of mucin outputs.

The approach used in this study for the determination of mucin, was similar to that used by Lien et al. (1995, 1997), who employed the large difference in carbohydrates composition between gastric and intestinal mucins, particularly with respect to the GlcNAc/ GalNAc ratios. Gastric mucin has a higher GlcNAc content relative to GalNAc, where the GlcNAc/ GalNAc ratio has been found to be 2.35 for pig gastric mucin (Scawen & Allen 1977) and 0.55 for pig small intestinal mucin (Mantle & Allen 1981, Allen 1981). In this experiment the GlcNAc/ GalNAc ratios in crude mucin and digesta among dietary treatments ranged from 0.55 to 0.98 (Table 4.3.3). These GlcNAc/ GalNAc ratios suggest a higher contribution of GalNAc relative to GlcNAc, indicating that crude mucin and total mucin originated primarily from the small intestine of the pigs. Similar carbohydrate ratios were observed by Lien et al. (1997, 2000) for growing pigs (55 kg) fed protein-free and fibre containing diets.

The crude mucin preparations in this experiment consisted primarily (> 80%) of the characteristic sugars of mucus glycoprotein, namely fucose, galactose, GalNAc and GlcNAc (Table 4.3.3), which were similar to findings from Lien et al. (1997) and Montagne et al. (2000). The high concentrations of mucin-type carbohydrates in crude mucin are characteristic for mucin-type glycoprotein, and would indicate the presence of oligosaccharides O-linked to serine and threonine residues (Montagne et al. 2000). In this experiment, we found that in contrast to crude mucin, in which the mucin carbohydrates represented over 80% of the total carbohydrates, mucin carbohydrates in ileal digesta represented under 10% of the total carbohydrates (Table 4.3.3). Lien et al. (1995, 1997) also obtained similar results from their work with pigs given amino acid-infused and saline-infused protein-free diets. Therefore, total mucin output was determined from the GlcNAc/ GalNAc ratio in crude mucin and the GalNAc content of ileal digesta, assuming all GalNAc in ileal digesta was associated with mucin (Lien et al. 1995, 1997).

Barley-based diet elicited the greatest daily total mucin and crude mucin output in the growing pigs, when compared to the six other dietary treatments used in this experiment (Table 4.3.1 and Table 4.3.2). From our crude mucin data we found that the 15% barley diet increased crude mucin output in growing pigs by 49% compared to the casein-based control diet. Relative to the control diet, the 10% canola meal and 10% wheat bran diets increased crude mucin output by 5% and 32%, respectively. The 0.3% lectins protein (1.5% kidney beans) diet increased crude mucin output in growing pigs by 38% relative to the casein-control diet, while crude mucin output was not different between the 2% tannins and control treatment groups. With the exception of lignin-

based treatment, the total mucin output values (Table 4.3.2) although smaller, followed the same pattern as with crude mucin.

Effects of ANF on Mucin Output

In this experiment, the concentration of crude mucin (g crude mucin per g ileal digesta) was significantly greater for the casein-based control (6.35%) and the lignin treatment (8.64%) relative to all other dietary treatments used in this experiment (Table 4.3.1). The protein status of the animal has been argued to influence the solubility of mucin in ileal digesta. Lien et al. (1997) found that crude mucin was 24% higher from amino acid –infused compared to saline-infused pigs fed a protein-free diet, while the difference in total mucin output between the two groups was only 6%, indicating the presence of other molecules in the crude mucin fractions. In our experiment, when digesta and crude mucin were corrected for digesta flow (using chromic oxide marker and feed intake), animals fed the control diet produced the least amount of crude mucin per day and total mucin per day. This result was expected because the control diet is a semi-synthetic diet with free amino acids and casein as the only protein source. Such a purified diet should have minimal abrasive impact on the intestine, resulting in less removal of existing mucus and less need for the lubricating effects of mucin secretions.

In this experiment, the fibre content, particularly the hemicellulose content may be the main contributing factor to the higher mucin output observed for barley-based and wheat bran-based treatments. Lien et al. (1995, 2000) found that in humans with ileostomies, the consumption of 1.1 to 33.7 g soy fibre/d in total enteral nutrition formulas increased daily ileal mucin output. They also observed that in growing pigs (55 kg), the consumption of 0 to 240 g pea fibre/d resulted in linear increases in the daily output of ileal mucin. Pea fibre and soy fibre are predominantly insoluble cellulose fibre (Lien et al. 1995, 2000). In the present experiment the cellulose contents among dietary treatments were similar among barley, canola meal, wheat bran and the control diets (Table 4.2.5) and therefore could not explain the higher mucin output in barley and wheat bran treated pigs relative to canola meal or to casein-based control treated pigs. However, there were significant differences in the hemicellulose content among these dietary treatments, with barley and wheat bran treatment containing the highest hemicellulose contents.

The response of the intestine to soluble fibre appears to increase the rate of cell replacement as opposed to the rate of mucin secretion (Lien 1995). It has been suggested that the intestine responds to the consumption of soluble fibre (eg beta-glucan and pectin) by increasing crypt cell

production rates (Jin et al. 1994). Crypt cell production rates are increased in response to localized cell damage (Rijke 1976). With the replacement of intestinal cells, the mucosal barrier would presumably be re-established and maintained by mucin flow from newly differentiated goblet cells and the secretion of mucus from the the crypt cells themselves (Lien et al. 2000). These differences are not reflected in the measures of mucus secretion with soluble fibre versus insoluble fibre (Satchithanandan et al. 1989, 1990). Thus, the findings of this experiment add further support to the argument that insoluble fibre, not soluble fibre, has the greater impact on measurable mucin secretion in the gastrointestinal tract of the growing pig.

Kidney beans (1.5%) in the diet elicited similar mucin output in growing pigs as 10% wheat bran (Table 4.3.1 and Table 4.3.2), even though the fibre content, including hemicellulose, of the kidney beans diet was substantially lower than that of the wheat bran treatment (Table 4.2.5). Perhaps the crude mucin output observed with the kidney beans treatment could be attributed to the lectins content of the beans. Lectins from kidney beans have been shown to be a potent stimulator of both cell proliferation and mucin secretion (Pusztai et al. 1988, Schulze et al. 1995). Thus, while most legumes are excellent sources of protein, caution should be taken when including them in the diets of growing pigs, because legumes that contain high lectins content could potentially inhibit growth in swine. However, we did not observe any significant negative effects on growth, feed efficiency or nitrogen balance in growing pigs fed the kidney beans dietary treatment, which may be due to the inclusion level in the diet.

Orally-administered hydrolysable tannins has been reported to cause hypersecretion of mucin and erosion of the mucosa of the stomach and duodenum including excess sloughing of the mucosa of the esophagus and colon (Vohra et al. 1961, Mitjavila et al. 1977). However, results with condensed tannins are more variable (Sell et al. 1985, Jansman et al. 1993b). In this experiment we saw that the inclusion of 2% condensed tannins (from grape) in the diets of growing pigs for two weeks did not affect growth, feed efficiency, mucin output, or nitrogen balance. Studies in rats using condensed tannins of sorghum also showed no effect on growth and only slight elevation of mucin secretion (Sell et al. 1985). Studies in piglets (Jansman et al. 1993a & b), as with our results, observed no effect of condensed tannins on mucin secretion. Therefore, it can be concluded that condensed tannins does not exert any observable anti-nutritional effects in pigs through increased mucin secretion.

Amino Acid Digestibility and Availability in Feedstuffs

There are various ways of describing amino acid digestibility. When ileal amino acid flows are not corrected for the endogenous amino acid flows, the digestibility coefficient is described as 'apparent'. However, if the ileal flow is partly corrected for the actual endogenous amino acid flow, using some basal estimate of endogenous loss, then the coefficient may be referred to as standardized true digestibility or 'standardized digestibility' (Moughan 2003). Standardized digestibility infers that digestibility coefficients have been standardized within the study to give a 'basal' endogenous ileal amino acid loss (Moughan 2003). Thus, in this experiment because all the diets were casein-based with all six ANF diets having similar casein content as the control diet, we were able to report both apparent and standardized protein and amino acid digestibility.

Endogenous nitrogen and amino acid loss from the small intestine has traditionally been determined after feeding the animal a protein-free diet. However, this method has been criticized for creating a physiologically abnormal state (Low 1980). Researchers (Moughan & Rutherford, 1990, Butts et al. 1993a & b) have shown that the presence of dietary protein or peptides in the gastrointestinal tract results in increased loss of endogenous nitrogen and amino acids from the small intestine of the growing pig compared to a protein-free diet. Under most conditions, nutritionists assume that the endogenous nitrogen loss (which includes mucin, along with sloughed cells, enzymes, and other intestinal secretions) by an animal is relatively constant (Furuya & Kaji 1992). However, if the diet contains components that stimulate mucus production then it is possible that the amount of mucin loss may be a function of the diet.

The casein-control diet in this experiment was used as a basal diet to estimate endogenous protein (and amino acid) output. Diets with highly digestible protein sources, such as casein, have been used to determine endogenous protein and amino acid loss in pigs (Kies et al. 1986, Furuya & Kaji 1989, also see review by Jansman et al. 2002). It can be assumed that the true digestibility of crude protein and amino acids in casein is 0.99, and that casein does not induce any specific secretion of endogenous protein and amino acids (Jansman et al. 2002, Grala et al. 1998, Kies et al. 1986). With these assumptions, the amount and amino acid composition of basal endogenous crude protein can be calculated from studies in which the apparent ileal digestibility of casein has been determined (Jansman et al. 2002, Kies et al. 1986, Furuya & Kaji 1989). Standardized and apparent ileal digestibility values of protein (nitrogen) and amino acids for the casein-control diet from the present experiment were in agreement with results reported by other researchers (Kies et al. 1986, Furuya & Kaji 1989, Chung & Baker 1992).

In the case of amino acids, ileal digestibility is taken as the basis for calculating supply to the body, because nitrogenous compounds absorbed in the large intestine of pigs do not contribute significantly to body supply (Zebrowska et al. 1978). Values for apparent and standardized digestibilities of nutrients are affected by levels of feed intake and by the diet characteristics (Souffrant et al. 1993, Sauer et al. 2000). Since the levels of feed intake were not found to be significantly different ($p = 0.52$, Table 4.3.16) among dietary treatments, therefore any changes in digestibilities must be due to diet characteristics. As shown in the digestibility results in Table 4.3.6 and in confirmation with the works of others (Stein et al. 1999, Sauer 1986); endogenous amino acid losses were higher for threonine. These data suggest that a greater proportion of this amino acid is required for maintenance in the growing pig, which supports data by de Lange et al. (1995) who reported that when endogenous N losses result in substantial losses of limiting amino acids, the amino acid and energy requirements for maintenance were also increased.

Sève and Hess (2000) discussed dietary conditions that influence the levels of endogenous loss; primarily, the rate of endogenous secretion was dependent on the amount of protein and fibre in the diet. From our results, we saw that specific ileal flows of protein and amino acid are affected by specific dietary components such as dietary fibre (in agreement with the work of Schulze 1994, 1995a), lectins (Schulze et al. 1994), and condensed tannins (Jansman et al. 1995). It has been concluded that the length of the pre-test period may affect the amount and amino acid composition of endogenous crude protein in ileal digesta of pigs (Jansman et al. 1993a Leterme et al. 1992). In this experiment the pigs were subjected to the dietary treatments for 12 days prior to digesta collection. Growth rate in this study showed noticeable differences beginning one week on the dietary treatments (see Figure 4.3.8). This result indicates that the time of adaptation was sufficiently long to elicit diet-specific responses.

Barley and wheat bran dietary treatments were noted to have the lowest apparent and standardized amino acid digestibilities in this experiment (Table 4.3.5 and Table 4.3.6). The digestibilities and ileal amino acid losses results (Table 4.3.7) observed in this experiment would suggest that wheat bran and barley increase endogenous protein and amino acid losses, probably through an increase in mucus secretion. Lien et al (1995) determined that mucin represented approximately 5 to 11% of total endogenous protein. The highest amino acid concentrations in mucin were threonine (28-35%), serine (13-16%) and proline (7-24%). The higher protein (N) and threonine endogenous losses observed for wheat bran and barley treated pigs would therefore

indicate an increase in mucin loss. In mucin, threonine is the largest contributor of the indispensable amino acids; however, its contribution in non-mucin endogenous protein is considerably less (Lien et al. 1995, Montagne et al. 2000, Mantle & Allen 1981). Thus, an increase in the level of threonine, relative to the contents of other amino acids in digesta at the terminal ileum would imply an increase in mucin secretion.

While the condensed tannins in our dietary treatment showed no effect on mucin secretion in the growing pig, it did reduce nutrient digestibilities. Similar findings were noted by Jansman et al. (1993), when they fed piglets field beans with various levels (0.4%-1.4%) of condensed tannins. In the present study, condensed tannins (2%) from grape, in the diet of growing pigs reduced apparent and standardized protein (nitrogen) and amino acid digestibilities (Table 4.3.5 and Table 4.3.6). Various studies (see extensive review by Jansman 1993) have suggested that tannins in different feedstuffs reduced apparent protein and amino acid digestibilities in pigs and other animals. These reduced protein and amino acid digestibilities do suggest that tannins have some anti-nutritional effects in growing pigs. However, in our experiment, tannins did not have any significant effect on weight gain, feed efficiency or nitrogen balance, suggesting that perhaps condensed tannins from grapes had no specific affect on protein metabolism or utilization, apart from reducing digestibilities of amino acids in tannins-based feed (Griffiths 1989). The main effects of tannins have been suggested to be attributable to their protein-binding capacity (Jansman 1993). The reduced protein and amino acid digestibilities resulting from the inclusion of condensed tannins in the diet of pigs may be due to interaction of tannins with proteins that originate from dietary sources or from endogenous intestinal secretions (Griffiths 1989, Jansman 1993). The predominant mechanisms are said to be hydrogen bonding between the phenolic groups of condensed tannins with the keto-imide groups of proteins, and additionally, hydrophobic bonding resulting from the co-alignment of the phenolic groups in tannins and the aromatic side chains found in proteins (Griffiths 1989, Bravo 1998). The reduced protein and amino acids digestibilities noted with this dietary treatment could not be explain by increased mucin secretion because we observed no increase in mucin secretion or ileal amino acid loss from the addition of condensed tannins in the diet of growing pigs.

Gastrointestinal Tract and Internal Organs

The small intestine can be segmented into distinct regions: duodenum, jejunum and ileum. Though we did not perform histological staining in the intestine, we quantified mucosal weght in

the segments of the intestine. Montagne et al. (2000) have shown that the concentration and flow of mucin protein increased linearly along the small intestine (from duodenum to ileum) in pre-ruminant calves. Because intestinal mucin is not digested prior to the large intestine (Hoskins 1984, van der Schoor et al. 2002), mucin protein in ileal digesta represents mucin from the entire gastrointestinal tract up to the terminal ileum. Lien et al. (1995, 1997) has shown that 27% of the total ileal loss of mucin protein in growing pig was of gastric origin; a similar proportion was observed in the pre-ruminant calf (Montagne et al. 2000). These results by Lien et al. (1995, 1997) and Montagne et al. (2000) would suggest that the majority of the mucin loss at the ileum is primarily from the small intestine. Several researchers (Vahouny & Cassidy 1986, Satchithanandam, 1990) have shown that high fibre diets induce structural and morphological changes that are indicative of a capacity for higher mucin secretion in the digestive tract. Ileum total weight/length and ileum mucosa weight/length were greater in wheat bran relative to control ($p<0.05$). These ileum data provide evidence to support the fact that wheat bran increases mucus secretion and therefore may increase dietary threonine requirement.

Though not significant, canola meal, barley and wheat bran treated pigs tended to have larger stomach specific weights (i.e., weight stomach per body weight). Additionally, canola meal-and wheat bran-treated pigs showed trends ($p=0.08$) towards larger small intestine specific weights (Table 4.3.11). The tendency towards larger stomach and small intestinal specific weights in these animals may be due to the bulkiness caused by the fibre content in the diet. Canola meal, barley and wheat bran diets had more fibre than the other dietary treatments used in this study. Work by Stanogias and Pearce (1985) had found significant effects of both type and level of dietary fibre on the wet empty weight of the stomach, small intestine and proximal colon of pigs. They stated that the empty weight of various segments of the gastrointestinal tract was related to the labour performed by the digestive tract. Thus, in this experiment the higher fibre diets meant the gastrointestinal tract had to work harder and this workload is reflected in the weights.

There are two main factors that could contribute to greater grastrointestinal weights caused by high fibre diet: increases in mucosal and/or muscularis weights. The effect of the canola meal and wheat bran treatments on the small intestine can be attributed to changes in the mucosa weight as noted by the elevation in mucosal weight in certain segments of the small intestine (Table 4.3.12). Because we did not measure the mucosa or muscularis of the stomach we can only speculate that the larger stomach weights seen with these treatments is due to a combination of both elevations of mucosa and muscularis weights due to the strain from the fibre in the diets.

Lignin treatment used in this experiment was observed to significantly impact the large intestine of growing pigs. The large intestine weight and length (corrected for body weight) were significantly higher for lignin treatment than for any other dietary treatment (Table 4.3.11 and Table 4.3.12). The speculation is that the lignin treatment led to higher microbial (i.e, protozoa) activities in the large intestine of these animals. The increase in microbial activity in the lignin-treated pigs would be directly related to the lignin in the diet, which was probably being utilized as a primary energy source for the protozoa. The extremely low fecal fibre digestibility values (Table 4.3.10) observed for the lignin treatment provide some support in favour of this argument.

Dietary treatments used in this study did not appear to have any severe systemic effects on growing pigs. This was concluded from the similarities in specific weights (Table 4.3.14) of the liver, spleen and kidneys in pigs fed the various dietary treatments in this study. This result was expected because the diets were formulated to provide the dietary treatments within a physiologically relevant range. There have been several studies indicating that hydrolysed tannins may cause systemic toxic effects (Mitjvaila et al. 1977, Boyd 1973), however, it is less clear if condensed tannins can cause systemic effects. The results of this study as well as other studies (see review by Jansman 1993) would suggest that a low level (<2%) of condensed tannins in the diet has no systemic effects on growing pigs.

There have been studies in pigs showing that the inclusion of kidney beanss (*Phaseolus vulgaris*) can significantly lower the weights of the pancreas, liver and spleen (Huisman et al. 1990). However, in all these studies, inclusion level of kidney beans in the diet was 20% or more. Because the inclusion level of kidney beans in this study was 1.5%, we did not observe a similar finding. So there would appear to be a threshold level of kidney beans in the diet necessary to trigger systemic effects in pigs.

Plasma Amino Acid Concentrations

Cystine, glycine and histidine were all observed to be significantly different, usually with barley and lignin treatments having the lowest values relative to the control treatment. This may be related to the lowering of (although frequently not significant) overall dietary protein digestibility caused by ANF in barley and lignin dietary treatments.

Growth Performance and Nitrogen Balance

Young pigs' intestinal enterocytes have a lifespan of approximately 2-5 days (Patience et al 1995). Thus, 7 days of adaptation to the test diets prior to sample collections for nitrogen balance were sufficient for any treatment effects to be expressed. There were no significant differences in feed intake among the groups primarily because feed intake was restricted to 5% daily body weight. As shown in Figure 4.3.8, during the first week on the dietary treatments, all animal experienced similar body weight gain; however, by the second week on the treatments, pigs fed wheat bran and barley treatments had lower body weight gain. Despite the lower body weight gain seen for the animals fed wheat bran and barley-based treatments there were no significant differences in gain-to-feed coefficients among treatment groups. But, based on the crude mucin output, ileal nutrient loss and apparent amino acid digestibility results, we could speculate that because of the greater demand for amino acids for mucin synthesis there were less amino acids available for protein deposition. Thus, explaining the lower weight gain observed in the animals fed the barley and the wheat bran diets compared to those fed the control diet.

Pigs receiving the wheat bran and the barley dietary treatments had lower nitrogen retention than all other treatments, except for the lignin treatment. The lower nitrogen retention for wheat bran and barley treatments could be a result of the greater endogenous protein losses, which would affect whole body protein retention and deposition. Souffrant et al. (1993) and Krawielitzki et al. (1994) showed that approximately 25% of total endogenous protein secretions are present in the ileal digesta of pigs. This endogenous nitrogen is then replenished at the expense of amino acids for growth (Fuller & Reed 1998).

Because the pattern of amino acids required for maintenance is different from that required for growth (NRC, 1998), replenishing endogenous nitrogen losses will alter the pattern of amino acids available for protein deposition. This shift in patterns may in turn result in additional urinary nitrogen excretion (Seve & Henry 1996). Thus, an increase in endogenous mucin losses would result in less amino acids such as threonine, proline, and serine being available for nitrogen retention. If any of these amino acids becomes limiting, then other amino acids would be in excess resulting in higher urinary nitrogen losses. So the combination of higher endogenous losses of nitrogen via the feces and urine would lead to lower nitrogen retention. Therefore, the significantly lower nitrogen retention observed with wheat bran and barley treated pigs could be a consequence of greater mucin excretion in these pigs.

4.5 Conclusions

Anti-nutritional factors in pig feedstuffs impact mucus secretion. Because the mucin protein in mucus, which is rich in threonine, is not reutilized by the animal, this increase in mucin secretion may affect threonine loss from the animal, threonine availability to the animal, and hence threonine requirement of the animal. The results of this experiment clearly showed that ANFs increase ileal mucin output, ileal threonine flow, and decrease ileal threonine digestibility. We have showed that a 15% barley diet increased crude mucin output in growing pigs by 49% compared to a casein-based diet, while 10% canola meal and 10% wheat bran diets increased mucin output by 5% and 32%, respectively. The 0.3% lectins protein (1.5% kidney beanss) in the diet also increased mucin output by 38%, but 2% tannins had no effect on mucin output. These data were supported by similar trends that were noted among dietary treatments for threonine and protein ileal flow and ileal digestibility. Threonine ileal flow for barley treated pigs was 119% greater than the control treatment, while wheat bran was 95% greater; tannins and kidney beans were 74% and 72% greater, respectively. Ileal threonine flow from canola meal treated pigs was 58% greater than control while lignin was only 11% greater than control. A similar trend was observed for ileal protein flow, where barley-treated pigs had the greatest output and lignin-treated pigs the lowest. Ileal digestibility data showed that the barley-based treatment lowered ileal threonine and protein digestibility by 12% and 8%, respectively, compared to the casein-control dietary treatment. Wheat bran had the next lowest ileal threonine digestibility of 11% lower than the control treatment, while protein digestibility was 10% lower than control. Besides mucin flow there were various other parameters that were measured in this experiment. Collectively these data all support the main finding from the mucin flow results, namely that of the six ANF diets examined, 15% barley not only created more mucin output and threonine loss, but barley also affected nitrogen retention. Therefore, barley was chosen as the ingredient for the subsequent experiment to test whether increased mucin production increases the threonine requirement of pigs.

Chapter 5
COMPARISON OF THREONINE REQUIREMENT IN GROWING PIGS FED A CASEIN-BASED OR BARLEY-BASED DIETS

5.1 Introduction

The gastrointestinal tract is exposed to all the physical and chemical forces of digestion. The gastrointestinal tract is covered with a protective layer of mucus whose primary function is to protect the underlying epithelial cells from digestive processes (Neutra & Forstner 1987, Toribara et al. 1993). The major component of mucus is a glycoprotein known as mucin. Mucin has a protein core which usesprimarily threonine as an anchor for the attachment of carbohydrate side chains (Allen 1981, Forstner & Forstner 1986). The threonine in mucin is passed into the colon and is not available for reuse by the animal (Hoskins 1984, van der Schoor et al. 2002). This may explain why the gastrointestinal tract uses 60% of dietary threonine (Bertolo et al. 1998, Stoll et al. 1998). The presence of anti-nutritional factors (ANF) in feedstuffs is a major concern since some of these ANF have been shown to increase mucus secretion (Pusztai et al. 1982, Jansman 1993, Satchithandam et al. 1990). Increased mucus secretion may cause threonine, which is the second limiting amino acid in most cereal grains products that are fed to swine (NRC 1998), to become a more limiting amino acid in regards to the animal's development.

In the previous experiment (Chapter 4) we established a link between ANF in pig feedstuffs, ileal mucin output and ileal threonine loss. That experiment served as a basis for this current experiment: to determine if increased mucin output results in an increased threonine requirement for pigs. Core mucin protein from the major intestinal secretory mucus contains high quantities of threonine, proline, and serine (Allen 1981, Lien et al. 1997). This protein core is not recycled for use by the animal (Hoskins 1984, van der Schoor et al. 2002) rendering a substantial part of the dietary threonine unavailable for protein synthesis. Therefore, mucus secretion should have a measurable impact on the animal's requirement for threonine. Evidence for this argument comes from several researchers who have shown that the portal appearance of dietary threonine, when expressed as a proportion of intake, is lower than that of other indispensable amino acids (see review by Reeds et al. 2000). Further evidence to support the idea that threonine requirement may be affected by intestinal mucin secretion came from the work of Zhao et al. (1986) and Bertolo et al. (1998). Zhao et al. (1986) conducted a study in adult humans, in which the body threonine

balance was calculated from the difference between threonine intake and oxidation. Zhao et al. (1986) concluded that there was an additional non-oxidative pathway of threonine loss from the body, because at very low intakes of threonine the subjects were in positive threonine balance (threonine oxidation was less than threonine intake) but at the same time they were in a state of negative nitrogen balance. Bertolo et al. (1998) found that threonine requirement of piglets was 60% lower when gut atrophy was induced. Thus, literature evidence as well as the results from our first experiment indicates that there may be a link between threonine requirement and intestinal mucin output.

In our first experiment we showed that ANFs in pig feedstuffs caused increases in mucus production and ileal threonine loss. In that experiment, we compared the mucin outputs of pigs fed a casein-based diet versus pigs fed the casein based diet plus lectins (as kidney beans), lignin, canola meal, barley, or wheat bran. From that first experiment, we observed that all the diets caused increases in mucin output when compared to the casein-based control diet. But the primary conclusion from our first experiment was that a barley-based diet (primarily insoluble fibre from barley) had the greatest impact on mucin output and ileal threonine output in growing pigs.

Thus, we hypothesized that the threonine requirement of swine would be affected by feedstuffs that increased gastrointestinal mucin output and ileal threonine output. To address our hypothesis, the overall objective for this experiment was to determine whether the addition of ANF, in this case insoluble fibre from barley, would lead to an increase in threonine requirement. Specifically, we wanted to determine whether there was a difference in threonine requirement for growing pigs fed a barley-casein-based diet compared to a casein-based diet.

The indicator amino acid oxidation (IAAO) method was used to determine threonine requirement for this experiment. This method was developed and validated in growing pigs (Kim et al. 1983 a & b, Ball & Bayley 1984) and has been shown to provide estimates of requirement which are similar to those determined by the classical nitrogen balance and growth techniques (Pencharz & Ball 2003). Because of the short adaptation time required between levels of the test amino acid, the IAAO technique allowed us to determine the individual threonine requirements on each of the diet treatments, within each animal, hence accounting for within animal variations.

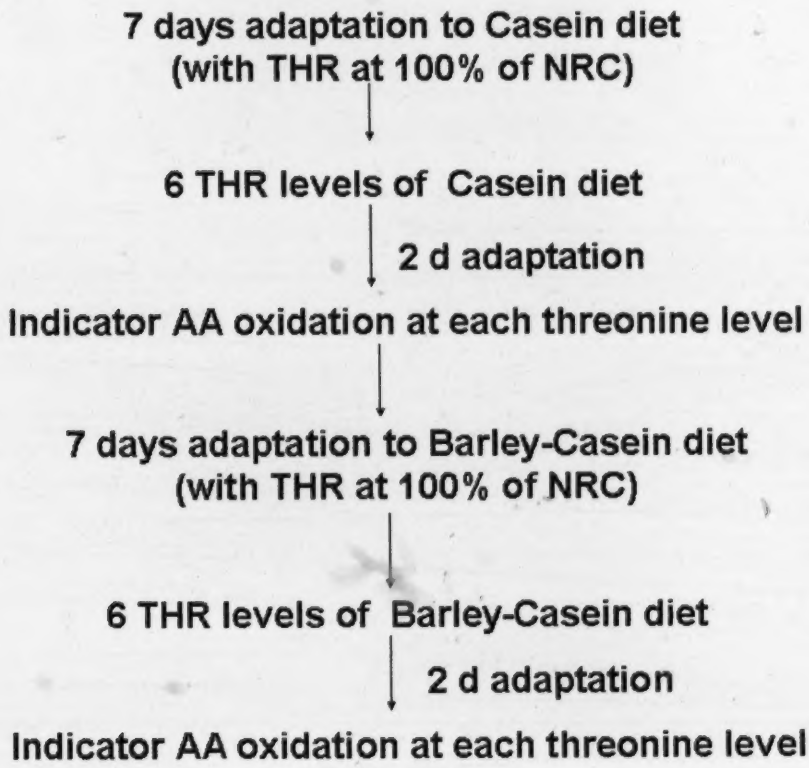
5.2 Materials and Methods

5.2.1 Experimental Design

Approval for this study was granted through the Animal Policy and Welfare Committee of the Faculty of Agriculture, Forestry and Home Economics, at the University of Alberta. Six barrows were obtained from the Swine Research and Technology Centre (SRTC) at 21 days old (7-10 kg). Pigs were housed together in groups of two for three to four days to minimize stress. During the group housing, they were gradually weaned from the starter diet (Table 5.2.1) to the casein-based experimental complete diet (Table 5.2.2), providing 100% of NRC (1998) requirement for total threonine (THR) for pigs 15 kg. Pigs were then housed individually and given 7 days further adaptation, to first a casein-based diet containing threonine at 100% of NRC requirement for pigs 15 kg followed by the indicator amino acid oxidation studies to determine the threonine requirements of the pigs. Each pig received each of the six casein-based diets, which differed only in threonine concentration (Table 5.2.3). There were two days of adaptations (Bertolo et al. 2001) to each of these threonine levels followed by measurement of phenylalanine oxidation using the IAAO method. Pigs were then adapted for 7 days to a barley-casein based diet containing threonine at 100% of NRC requirement for pigs 15 kg. Again, each pig was subjected to six barley diets (see Table 5.2.3), which differed only in threonine contents. Phenylalanine oxidation was measured at each diet level, with a minimum of two days adaptation time between dietary treatments. For casein and barley diets, all six threonine diets were offered sequentially in a randomized manner. Pigs were fed ad libitum during the weaning-adaptation period, and then fed at 14.70 MJ/ kg BW/day, which is equivalent to 95 % of predicted ad libitum intake.

The following flow chart summarizes the experimental design:

Figure 5.2.1: Experimental design for testing threonine requirement in growing pigs fed casein-based and casein-barley diets


7 days adaptation to Casein diet
(with THR at 100% of NRC)
6 THR levels of Casein diet
2 d adaptation
Indicator AA oxidation at each threonine level
7 days adaptation to Barley-Casein diet
(with THR at 100% of NRC)
6 THR levels of Barley-Casein diet
2 d adaptation
Indicator AA oxidation at each threonine level

5.2.2 Diet and Feeding

The dietary treatments consisted of six casein-based diets with different levels of threonine, and six barley-casein based diets also with six threonine levels (Table 5.2.3). The casein-based diet contained 12% casein and the barley–casein based diet contained 50% barley and 6% casein (Table 5.2.2). To minimize variation in other nutrients among experimental diets, dietary threonine concentrations were achieved by formulating and making casein- and barley-based diets with threonine concentrations at 60% and 150% of predicted requirement, and mixing these two diets in appropriate proportions (Table 5.2.3). Pigs were given *ad libitum* access to water via nipple drinkers. They were fed twice daily at 0700 and 1900 hr, with the exception of the oxidation days, where half the daily feed ration was divided into 16 portions, and fed every half-hour during oxidation; the remaining daily ration was fed in the evening. Any feed not eaten was collected, dried and weighed to obtain net feed intake. Diets were formulated to be isonitrogenous; using asparagine, and isoenergetic; using cornoil, cornstarch and sugar, and formulated to be first limiting in threonine, with all other amino acids provided at 150% of NRC for pigs 15 kg (see Table 5.2.2 to Table 5.2.6).

Table 5.2.1: Nutrient content of commercial starter diet for weaned pigs[1]

Diet Nutrient	Diet Amount
Crude protein	24.46 %
Digestible energy	3.61 Mcal/kg
Calcium	0.97 %
Phosphorus	0.77 %
Lysine	1.66 %

[1]Consultant Feeds, Calmar, Alberta, Canada, produced this diet. In with keeping with competitive privacy act, the above information is only information that they will divulge.

Table 5.2.2: Diet formulation (g/kg)

Ingredients	Casein-60[1]	Casein-150[2]	Barley-60[3]	Barley-150[4]
Barley	0	0	500	500
Casein	116.8	116.8	60.3	60.3
Corn oil	9.5	9	51.5	51
Cornstarch	302.53	300	0	0
L-Histidine	2.88	2.88	2.83	2.83
L-Isoleucine	4.53	4.53	4.78	4.78
Potassium chloride	6.1	6.1	1.7	1.7
L- Asparagine	57.2	53.5	47.7	44
L-arginine	4.03	4.03	2.46	2.46
L-Cystine	4.8	4.8	3.53	3.53
L-Leucine	7.37	7.37	7.27	7.27
L-Threonine	0	6.73	0	6.73
L-Tryptophan	1.9	1.9	1.79	1.79
L-Lysine HCl	12.7	12.7	14.62	14.62
DL-Methionine	1.46	1.46	1.77	1.77
Magnesium sulphate	1	1	0	0
Phenylalanine	5.32	5.32	4.3	4.3
Sugar	320	320	252.36	249.83
Solkafloc[a]	100	100	0	0
Thiamine	0.016	0.016	0	0
L-Tyrosine	0.13	0.13	1.39	1.39
L-Valine	5.72	5.72	5.7	5.7
Vitamin B_6	0.018	0.018	0	0
Vitamin-Mineral mix[b]	36	36	36	36
Sum	1000.00	1000.00	1000.00	1000.00

[1]Casein-60 and [3]Barley-60 are casein-based and barley-based diets that provided only 60% of total threonine requirement for 15 kg pigs as defined by NRC, 1998. [2]Casein-150 and [4]Barley-150 are casein-based and barley-based diets that provided 150% of total threonine requirement for 15-20kg pigs as defined by NRC, 1998. [a]Provided cellulose. [b] Provided the following per kg of vitamin mix: 21.5 g Ca; 8.4 g P_{dig}; 1g Mg; 0.20 g S; 595 mg Cu; 9.9 mg I; 7 g Fe; 1.6 g Mn; 7.4 mg Se; 3.5 g Zn; 15 mg biotin; 13 g choline; 80 mg folacin; 625 mg pantothenic acid; 950 mg niacin; 175 mg riboflavin; 880 ug vit B_{12}; 1550 mg vit.E; 300,000 IU vit.A; 35,000 IU vit.D.

Table 5.2.3: Mixing proportions of 60% and 150% threonine diets to achieve experimental diets containing 6 levels of threonine

% THR in experimental diets	THR (g/kg) in Casein diets	THR (g/kg) in Barley diets	Experimental diets[1]	% of THR-60 diet[2]	% of THR-150 diet[3]
60	0.411	0.397	C-60/B-60	100	0
80	0.558	0.544	C-80/B-80	78	22
100	0.711	0.697	C-100/B-100	55	45
110	0.777	0.764	C-110/B-110	45	55
130	0.924	0.911	C-130/B-130	23	77
150	1.077	1.064	C-150/B-150	0	100

[1]In this experiment there was a total of 6 casein-based diets each containing a different level of threonine (60, 80, 100, 110, 130 and 150% of total threonine requirement for pigs 15 kg). There were also a total of 6 barley-based diets with the same threonine contents as the casein diets. Therefore, C and B represent the type of diet and the numbers represent the threonine level of that diet.

[2]THR-60 diet is a casein-based or barley-based diet that provided 60% of total threonine requirement for 15 kg pigs as defined by NRC, 1998.

[3]THR-150 diet is a casein-based or a barley-based diet that provided 150% of total threonine requirement for 15 kg pigs as defined by NRC, 1998

Table 5.2.4: Nutrient and energy content of diets, calculated

Nutrient	Units	Casein-60[1]	Casein-150[2]	Barley-60[3]	Barley-150[4]
Ash (minerals)	%	4.69	4.69	5.28	5.28
Crude Protein	%	22.41	22.41	22.41	22.41
Dry matter	%	95	95	95	95
Ether extract (crude fat)	%	1.06	1.01	5.63	5.63
ME	KJ/kg	14.70	14.71	14.70	14.71
ME	Kcal/kg	3511.10	3513.48	3511.10	3513.48

[1]Casein-60 and [3]Barley-60 are casein-based and barley-based diets that provided 60% of total threonine requirement for 15 kg pigs as defined by NRC, 1998. [2]Casein-150 and [4]Barley-150 are casein-based and barley-based diets that provided 150% of total threonine requirement for 15 kg pigs as defined by NRC, 1998. ME= metabolizable energy.

Table 5.2.5: Nutrient and energy content of diets, analyzed

Nutrient	Units	Casein-60[1]	Casein-150[2]	Barley-60[3]	Barley-150[4]
Ash (minerals)	%	3.49	3.20	4.51	5.10
Crude Protein	%	20.34	20.18	20.05	20.84
Dry matter	%	93.37	94.55	92.81	92.59
Ether extract	%	1.31	1.31	6.70	6.47
GE	Kcal/kg	3905.89	3966.90	4148.30	4151.53
DE[5]	Kcal/kg	3609.37	3634.10	3592.34	3540.81
DE	KJ/kg	15.10	15.21	15.03	14.82
ME[6]	Kcal/kg	3478.16	3503.17	3463.82	3408.60
ME	KJ/kg	14.55	14.66	14.49	14.26
Fibre components					
NDF[7]	%	6.62	7.49	10.63	11.33
ADF[8]	%	3.23	4.55	2.80	3.03
Lignin	%	0	0	0.40	0.54

[1]Casein-60 and [3]Barley-60 are casein-based and barley-based diets that provided 60% of total threonine requirement for 15 kg pigs as defined by NRC, 1998. [2]Casein-150 and [4]Barley-150 are casein-based and barley-based diets that provided 150% of total threonine requirement for 15 kg pigs as defined by NRC, 1998. GE= Gross energy, determined by bomb calorimeter. [5]DE= digestibility energy. DE calculated from: DE= 949 + (0.789*GE) – (43*% ash)- (41*% NDF) (Noblet & Perez 1993) [6]ME= metabolizable energy. See **Equation 4.2.1: ME (May & Bell 1971):**,**Equation 4.2.2: ME (Noblet et al. 1989):** [7]NDF = neutral detergent fibre, includes cellulose, hemi-cellulose and lignin. [8]ADF= acid detergent fibre, includes cellulose and lignin.

Table 5.2.6: Amino acid composition of diets (%), analyzed[1]

Amino acids	Casein-60[2]	Casein-150[4]	Barley-60[3]	Barley-150[5]
Arginine	0.69	0.69	0.61	0.61
Cystine	0.53	0.53	0.47	0.47
Histidine	0.56	0.56	0.54	0.54
Isoleucine	0.96	0.96	0.95	0.95
Leucine	1.63	1.63	1.71	1.71
Lysine	1.73	1.73	1.65	1.65
Methionine	0.44	0.44	0.44	0.44
Phenylalanine	1.00	1.00	0.94	0.94
Threonine	0.41	1.08	0.40	1.06
Tryptophan	0.32	0.32	0.32	0.32
Tyrosine	0.58	0.58	0.58	0.58
Valine	1.19	1.19	1.16	1.16
Sum	10.05	10.71	9.76	10.43

[1]Degussa AG, Germany, performed amino acid analysis on diet samples (Llames & Fontaine 1994)

[2]Casein-60 and [3]Barley-60 are casein-based and barley-based diets that provide 60% of total threonine requirement for 15 kg pigs as defined by NRC, 1998. [4]Casein-150 and [5]Barley-150 are casein-based and barley-based diets that provided 150% of total threonine requirement for 15-20kg pigs as defined by NRC, 1998.

5.2.3 Oxidation Equipment

The oxidation equipment consisted of a respiration chamber, constructed around a steel and plexiglass metabolic crate (1.5m x 1.0m x 0.70m), through which air was drawn at a rate of 168 L/min by rotary vane pumps (Gast Model 1023, Benton Harbor, MI) so that the chamber was under negative pressure. The exiting air was pulled through a condenser to remove water vapour, split and metered (Canadian Meter Co. Inc., Cambridge, ON); CO_2 was collected quantitatively from approximately half the airflow in a series of five gas washing bottles, each filled with 100 mL of CO_2 absorber (monoethanolamine and ethylene glycol monomethyl ether: 1:2, v/v) (Caledon Laboratories Ltd., Georgetown, ON).

To ensure the complete collection of CO_2 at the time of the experiment, the oxidation equipment was calibrated prior to the first oxidation. The following procedure was as described by Bertolo et al (2001). During the equipment validation, a CO_2 analyzer (Beckman LB2, Beckman, Irvine, CA) was attached to this system to monitor CO_2 concentrations. To calibrate the equipment, a beaker with 3 L of sodium bicarbonate solution (1 mol/L) was placed inside the chamber on a stirrer; to this solution was added 1 μCi of $NaH^{14}CO_3$ (50 mCi/mmol, ARC, St. Louis, MO). Using a syringe pump (Ealing Scientific Ltd, Montreal, PQ), 3.5 mol concentrated hydrochloric acid was infused over 1 h to liberate all CO_2 from the solution. This procedure was designed to mimick the CO_2 excretion of a pig in the chamber. From the analyzed and collected CO_2, the percent recovery of CO_2 from the chamber was calculated to ensure appropriate airflow and to account for 100% of CO_2 leaving the system.

5.2.4 Indicator Amino Acid Oxidation Measurements

L- [ala-1-^{14}C] phenylalanine (55 mCi/mmol) in 0.01N hydrochloric acid was purchased from American Radiolabel Chemicals (ARC) Inc., St. Louis, Missouri. The priming and constant infusion rates for this experiment were derived from an earlier experiment, which studied the lysine requirement in growing pigs (Bertolo et al 2001). A prime: constant infusion ratio of 1.75 was found to be adequate for all pigs used in their study, which was also adequate for this experiment given the similarity in pig size between the two studies. For the oxidation studies, pigs were placed in the respiration chambers 0.5 h prior to the start of the isotope delivery to assure equilibration of CO_2 in the outflowing air from the chambers. The isotope was mixed with the feed and administered orally. Pigs were given a priming dose of 4.5 ml the tracer solution

(approximately 3.0mCi/L of L- [ala-1-^{14}C] phenylalanine) together with the first half hourly dose (1.5 ml) of the tracer solution. Thereafter, 1.5 ml of the tracer solution was given every half-hour in the feed during the four hours experiment. Breath samples were collected every half hour. The absorber was weighed, and sampled; from this sample, a 1ml aliquot was then mixed with Atomlight® scintillation cocktail (Packard Bioscience Co., Meriden, CT). The radioactivity of the breath samples was assessed using a liquid scintillation counter (Tricarb 4000, AMBAC Industries Inc., Downers Grove, Ill), and counted for 15 minutes or to an error of 2%. Counts were corrected for background and for counting efficiency by use of auto DPM mode on the scintillation counter. The total collected $^{14}CO_2$ as a percent of the dose of ^{14}C-phenylalanine (PHE) infused was plotted against time and the plateau was determined through visual inspection. From these values, the percent dose (labelled PHE) oxidized was calculated using ((DPM collected/fraction of the airflow collected)/ DPM infused) * 100%, over the plateau range. For the threonine experimental diets, the percent of dose oxidized of the labelled phenylalanine was plotted against the dietary threonine content.

5.2.5 Correction Factor For Increasing Background Radioactivity

In this experiment, each pig was subjected to at least twelve oxidations. Repeated isotopic tracer infusions result in an accumulation of ^{14}C-phenylalanine in the pig's body protein. Protein turnover releases this ^{14}C-phenylalanine which is then oxidized and released as $^{14}CO_2$, thereby creating a radioactive background. Experience has shown that direct measurement of this background prior to each isotope infusion is highly variable due to the increased activity of the animals at the start of an infusion study along with the initiation of the morning feeding regimen (Bertolo et al. 2001). Thus, radioactive background determination for this experiment was calculated based on experiments designed to measure the change in background radioactivity following repeated dosing and the subsequent decay curve (Bertolo et al 2001). Radioactive background was calculated from the following equation:

Equation 5.2.1: Radioactive ^{14}C background in expired breath of test animals

$$\text{Background (\% of infused dose)} = 0.3379 * e^{-0.1067 * \text{days since the previous study}}$$

5.2.6 Proximal Analyses

5.2.6.1 Fat, Energy, Ash, and Analytical Dry Matter

These analyses were performed in duplicate and according to AOAC (1984) methods.

Analytical dry matter was determined for feed samples. Samples (2 g each) were weighed and dried in a 110 ^{0}C oven for 5 h then re-weighed post drying. Ash analysis was also performed on these samples by placing in a 550 ^{0}C oven overnight and the residual weighed.

Gross energy of feeds were determined using Leco Bomb Calorimeter (AC-300, Leco Corp., St. Joseph, MI). This procedure is described in Chapter 4.

Crude fat (fat ether) was determined using Goldfisch extraction apparatus (Labconco Co., Kansas City, MO), as described in Chapter 4.

5.2.6.2 Nitrogen Analysis

Nitrogen contents of feed samples were determined by the method described by Sweeney and Rexroad (1987). This method involves sample combustion in an oxygen atmosphere followed by a series of catalytic reactions resulting in nitrogen gas production. Samples (100 mg portions) were weighed into tin foil and wrapped. Samples were then analyzed using LECO FP-428 Nitrogen/Protein Determinator (LECO Instruments Ltd., Mississauga, ON).

5.2.6.3 Fibre Analysis

Fibre analysis was performed using a modified method of Goering and van Soest (1970). This method is known as the filter bag technique (FBT) (Ankom Co. publication # 101, 1993). The procedure was described in Chapter 4.

5.2.6.4 Amino Acid Analysis of Diet

Amino acids in the diets were determined by Degussa AG (Hanau, Germany) by high performance liquid chromatography (HPLC; Llames & Fontaine 1994).

5.2.7 Statistical Analyses

Threonine requirements were calculated for both casein and barley dietary treatments. Dietary threonine intake (g/ kg BW) served as the main treatment effect. Threonine requirement within diets was established using a two-phase linear regression crossover model, as described previously (Kim et al. 1983a &b, Ball & Bayley 1984). Regression variables included the level of amino acid intake as the independent variable, and phenylalanine oxidation (% dose) as the dependent variable. This analysis works by partitioning the data points into two distinct regression lines. The data partition was chosen based on the model that produced the highest

regression coefficients for all dependent variables. The breakpoint represents the estimate for threonine requirement. Differences within the two dietary treatments were determined by GLM PROC MIXED model (SAS System V8, 1999). If the overall F-value of the GLM PROC MIXED model had a significance level of $p \leq 0.05$; then significant differences among treatments means were assessed by multiple comparisons using LSD procedure. Threonine requirements were also determined by fitting the data to a quadratic curve overlayed to the two-phase regression graph and establishing the first point at which the quadratic response curve intersected the plateau value established from the two-phase graph (Baker et al. 2002). The intercept of the quadratic curve and the plateau of the broken line have been proposed to better predict the estimate of the population (95%) requirement (Baker et al. 2002, Parr et al. 2003). The R-squares from the quadratic and the two-phase regression were used to determine the model that offered the best fit for the threonine requirement data.

5.3 Results

5.3.1 Growth Performance

Throughout the study all pigs remained healthy and interested in their environment. Average body weight at the start of the study period was 7.70 kg (SD 1.05 kg) and average final body weight at the end of the study was 27.77 kg (SD 3.13 kg). Average daily weight gain during the study was 0.47 kg/ day (0.23 kg SD), with an average daily gain of 0.26 kg (0.05 kg SD) on the casein diet and 0.68 kg (0.07kg SD) on the barley-based diet. As shown in Table 5.3.1, average weight gain and gain-to-feed within diet were the same; however, these values were different between diets with higher values for the barley-based diet.

Table 5.3.1: Weight changes, average feed intake and gain-to-feed in growing pigs fed either casein- or barley-based diets during an indicator amino acid oxidation study

Parameters (kg)	*Pig 3	Pig 4	Pig 5	Pig 6	Pig 7	Pig 8	Mean	Std dev
Initial BW	6.64	6.46	7.88	8.50	7.58	9.16	7.70	1.05
Final BW	32.35	30.90	26.10	25.00	27.35	25.00	27.78	3.13
Casein FI	0.43	0.41	0.47	0.52	0.48	0.58	0.48	0.06
Casein ADG	0.25	0.23	0.24	0.27	0.29	0.34	0.26	0.05
Casein G: F	0.61	0.56	0.56	0.57	0.60	0.58	0.58	0.02
Barley FI	1.08	1.04	0.90	1.05	0.96	1.06	1.02	0.07
Barley ADG	0.74	0.77	0.60	0.73	0.62	0.62	0.68	0.07
Barley G: F	0.70	0.84	0.70	0.75	0.68	0.62	0.71	0.07

*Pig 1 & 2 were used as preliminary pigs to ensure the pig would eat the diets during the experiment. [1] Body weights are given for the overall experiment showing the initial body weight of each animal on the morning just before they were placed on a test diet either casein or the barley-casein diet, and also for the final body weight for the last day on one of the experimental test diets. . BW= body weight, FI = feed intake, ADG = average daily gain, G: F = gain to feed.

5.3.2 Threonine Requirement

Phenylalanine oxidation was influenced by threonine intake (Figure 5.3.1through Figure 5.3.4). For the casein diet as threonine intake increased from 0.206 to 0.279 g THR/kg BW phenylalanine oxidation declined. Further increases in threonine intake from 0.355 to 0.539 g THR/ kg BW had no significant effect on phenylalanine oxidation. A similar oxidation pattern was observed between phenylalanine oxidation and threonine intake in the barley-casein treated pigs, where threonine intake from 0.199 to 0.349 g THR/kg BW caused a linear decline in phenylalanine oxidation, but increasing the threonine levels from 0.382 to 0.532 g THR/kg BW caused no further significant reduction in phenylalanine oxidation.

Threonine requirement on the casein and barley-casein diets for individual pigs are shown in Table 5.3.2. Within each diet the average of the individual requirements were used to express the overall dietary threonine requirement. Due to an inability to verify an oxidation plateau in the two-phase model, threonine requirements for two animals on the barley-casein diet were not used in determining the average requirement, that is only 4 pigs were used.

Table 5.3.2: Individual threonine requirement of growing pigs fed either a casein-based diet or a barley-casein based diet

Pig #	Casein diet (g THR/ kg BW)	Barley diet (g THR/ kg BW)
Pig3	0.355	0.313
Pig4	0.294	0.466
Pig5	0.348	-
Pig6	0.387	-
Pig7	0.388	0.386
Pig8	0.360	0.363
Mean	0.355	0.382
Std dev	0.034	0.064

The mean total dietary threonine requirement for 6 pigs fed the casein treatment was 0.355 (SD 0.034) g THR/kg BW and with upper and lower 90% confidence limits estimated at 0.319 g THR/kg BW and 0.391 g THR/kg BW, respectively. Summary of Table 5.3.2 showed that for pig 3, threonine requirement was lower by 15% on the barley diet compared to the casein diet. For pigs 7 and 8 threonine requirements were similar in values on both barley and casein diets. For the barley treatment, we were not able to estimate breakpoints for two of the six pigs because they did not exhibit a plateau in $^{14}CO_2$ in breath for enough THR intakes. The inability to establish plateau in $^{14}CO_2$ in breath also resulted in failure to obtain plateaus in the two-phase regression graphs for two of the six pigs. For the remaining four pigs, breakpoint estimates of threonine were 0.382 (SD 0.064) g THR/kg BW with upper and lower confidence limits of 0.281 g THR/kg BW and 0.483 g THR/kg BW, respectively. The overall mean threonine requirement between barley and casein diets showed 10% higher requirement from barley within repeated measure sensitivity of the method. The average differences (0.027 g THR/kg BW) in threonine requirement between the two diets showed that pigs required higher threonine intake when on the barley diet compared to the casein diet.

Figure 5.3.1: Threonine requirement by 2-phase linear regression based on phenylalanine oxidation as a percent of dose in pigs receiving several levels of threonine in casein-based diets

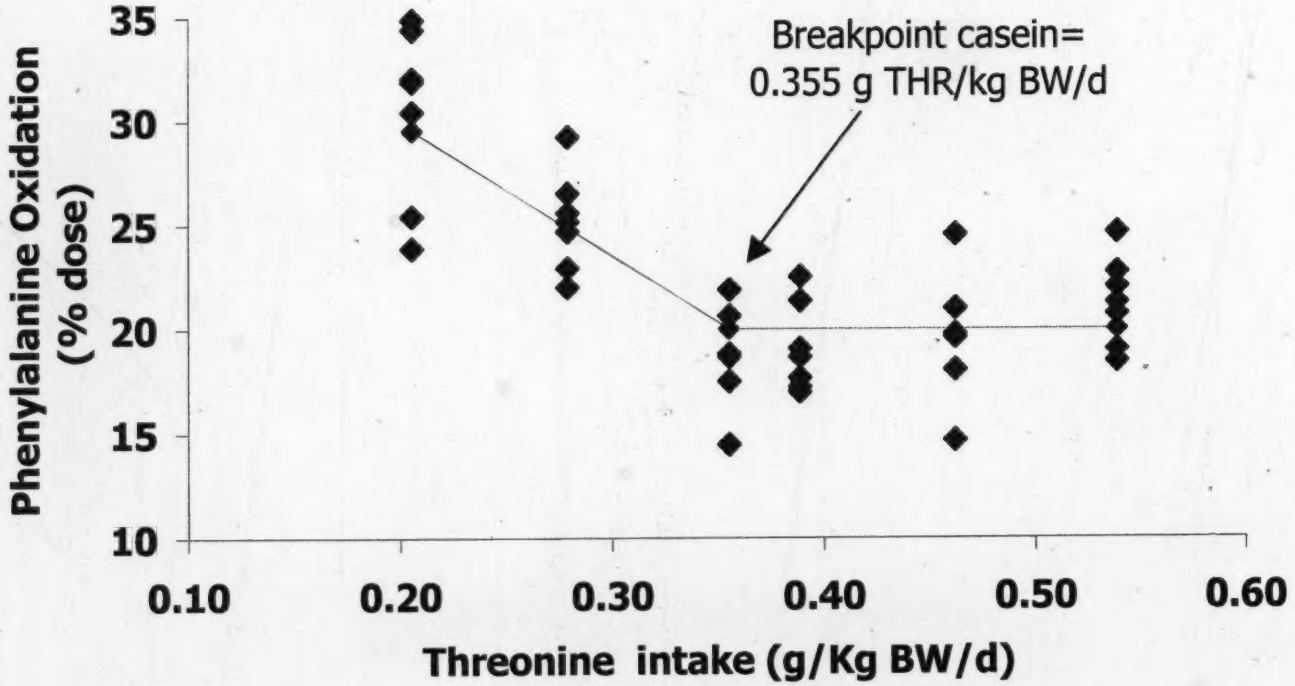


Figure 5.3.2: Threonine requirement by 2-phase linear regression based on phenylalanine oxidation as a percent of dose in pigs receiving several levels of threonine in barley-based diets

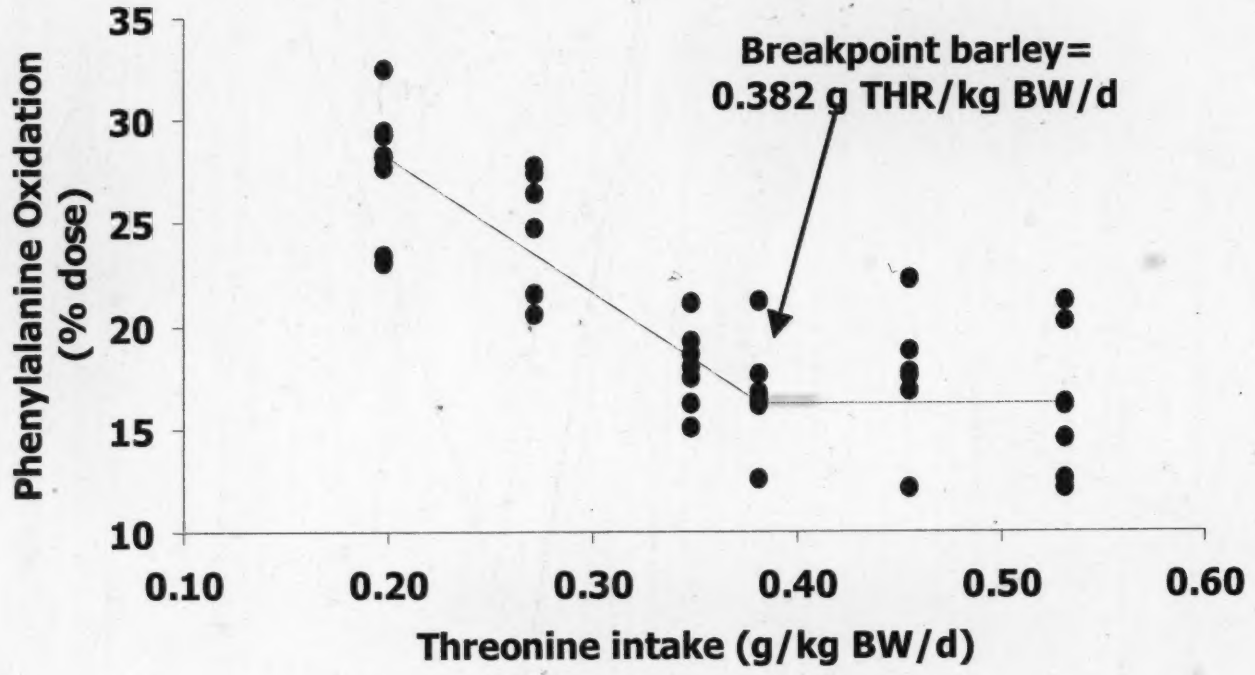
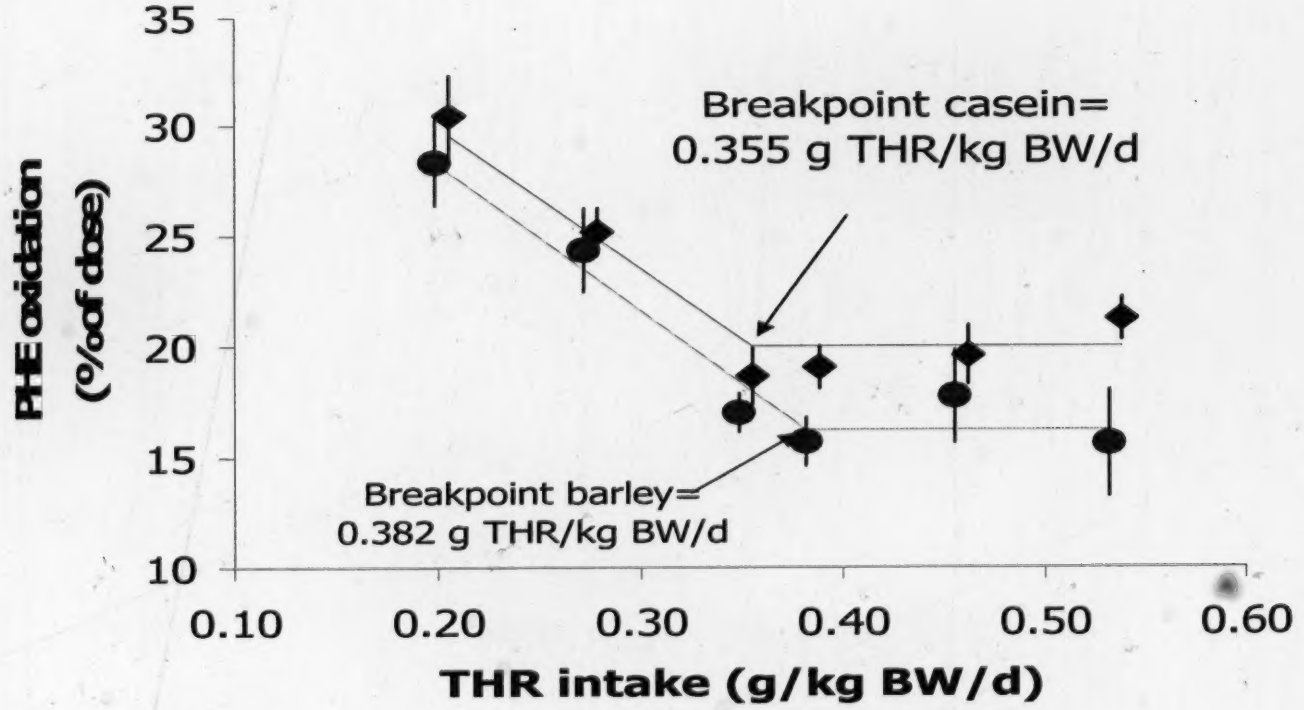


Figure 5.3.3: Threonine requirement by 2-phase linear regression. Mean phenylalanine oxidation as percentage of dose in growing pigs receiving six levels of threonine for both casein and barley diets



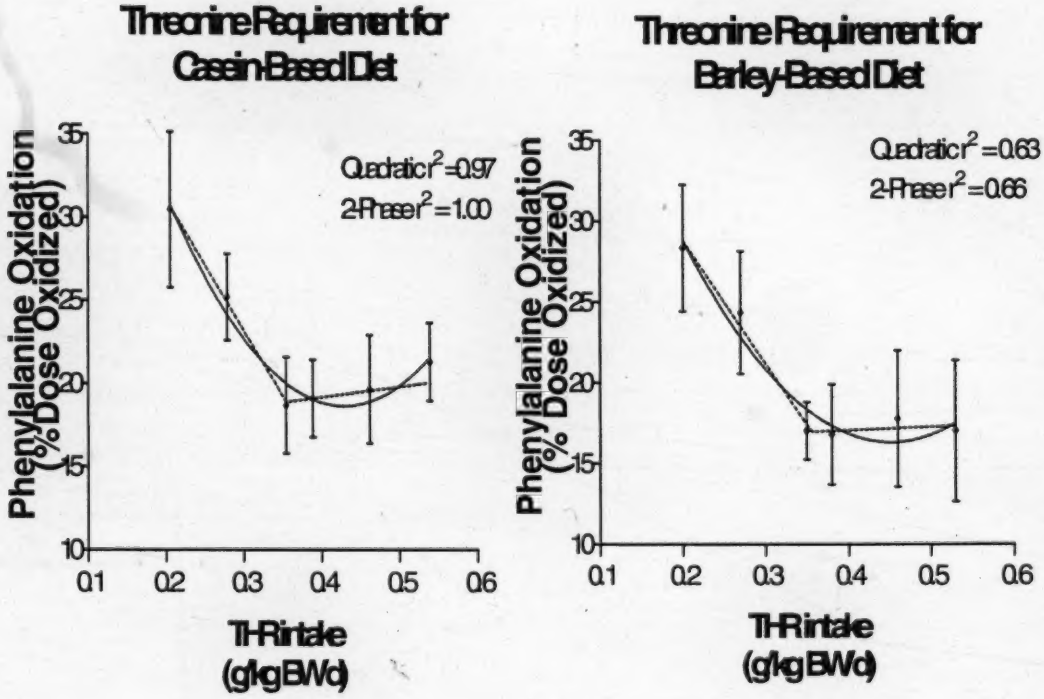
Because casein and barley are different in terms of threonine digestibility, one objective was to determine if the threonine requirement would change based on the digestibility factors for these diets (NRC 1998), i.e., when threonine requirement is expressed on total digestibility vs. apparent digestibility vs. true digestibility. When the dietary intake was expressed on total digestibility basis, differences between the diets are due to differences in feedstuff digestibility and endogenous losses. When formulated on ileal digestibility, differences between the diets are due to differences in endogenous losses. When calculated on true digestibility basis, values for threonine requirements for casein and barley diets should be the same. However, as shown in Table 5.3.3 these values were not the same; rather a lower value was noted for the barley diet compared to casein diet. Therefore, for barley, the values used for endogenous losses are underestimated. As shown in the threonine requirement figures in this chapter, despite the differences in requirement (regardless of the digestibility basis used in formulation) there were similar decline of oxidation in the barley and casein based diets, indicating similar utilization of the free threonine that was added to the diets.

Table 5.3.3: Threonine requirement of growing pigs fed either a casein-based diet or a barley-casein based diet as determined using different digestibility values

THR Digestibility	Casein (g/ kg BW/d)	Std dev	Barley-casein (g/ kg BW/d)	Std dev	p-value
Total	0.355	0.034	0.382	0.064	0.13
True	0.347	0.033	0.360	0.059	0.20
Ileal	0.312	0.030	0.324	0.052	0.31

For the casein diet, the 2-phase regression model predicted a break point of 0.355 g THR/kg BW. The threonine requirement was also fitted to a quadratic regression equation. Overlay of the two models (Figure 5.3.4) showed an upper estimate of the population threonine requirement (upper asymptote), was calculated to be 0.389 g THR/kg BW (n=6). For the barley diet, the 2-phase regression model predicted a break point of 0.382 g THR/kg BW. Overlay of the quadratic and the 2-phase model showed a threonine requirement of 0.402 (n=4).

Figure 5.3.4: Fitted broken-line and quadratic plot of phenylalanine oxidation as a function of threonine intake/ kg body weight for pigs fed casein-based and barley-based diet



A comparison of 2 methods for the statistical analysis of threonine requirement gave different values for pigs fed casein and barley diets (see Table 5.3.4). NRC (1998) predicts that the threonine requirement (based on total threonine) for pigs fed barley diet is lower than for those fed casein. However, both the two-phase regression and the quadratic fit model showed higher threonine requirement for pigs fed barley diet, in comparison to those fed casein diet.

Table 5.3.4: Total threonine requirement (g/kg BW) of pigs fed casein or barley-based diets, determined with different mathematical models.

Method	Casein		Barley	
	Mean	SE	Mean	SE
2-phase regression	0.355	0.01	0.382	0.03
Quadratic fit	0.389	0.02	0.402	0.03
NRC (total)	0.362	n/a	0.321	n/a

5.4 Discussion

There were two main factors that contributed to the threonine requirements estimated in this experiment: body weight and diet composition. For diet composition, the main difference between the diets used in this experiment was the presence of barley in the barley-casein diets. All diets in this study were formulated on total threonine digestibility, thus, accounting for the effect of diet ingredients digestibility on threonine requirement. Although not significant ($p>0.05$, Table 5.3.3), these threonine requirement results based on digestibilities showed that any differences in threonine requirement that were observed in this study would be due to endogenous secretion. A comparison of the oxidation graphs (Figure 5.3.3) for the casein and barley-casein diets showed similar decline of oxidation from both diets, indicating similar utilization of the free threonine that was added to the diets. Thus, any difference in requirement for this study should be due to difference in dietary ingredient between the diets, namely barley. For this study, the indication for threonine requirement based on endogenous secretion is in agreement with the increased mucus production and threonine losses that were noted during Experiment 1 for the comparison of casein versus barley diet.

Analysis of the individual threonine requirements suggested no difference in threonine requirement between casein and barley-casein diet (Table 5.3.2). However, due to the high errors in individual measurements relative to group measurements, most of the discussion focuses on group measurement. For the barley-casein diet, the data for two of the six pigs were not used in estimating threonine requirement because these animals did not establish plateau in expired $^{14}CO_2$.

Results from our experiment, in comparison to NRC, suggest that diet composition substantially impacts threonine requirement. According to the NRC (1998) computer program, the threonine requirement for the casein diet period should have been 3.66 g THR/d or 0.362 g THR/kg * d for the size of pig, and for the barley diet period the requirement should have been 6.45 g THR/d or 0.321 g THR/kg * d. A comparison of these NRC values to those determined in this experiment shows that during the casein diet period the threonine recommendation was equivalent to the NRC recommendation (0.355 vs 0.362 g THR/kg *d); however, during the barley diet period the threonine requirement determined from this experiment was about 20% greater (0.382 vs. 0.321 g THR/kg * d) than NRC (1998). Therefore NRC (1998) amino acid requirements underestimate threonine requirement when feeding a higher fibre (such as barley) diet.

Because it can be expected that the threonine requirement expressed as g/kg BW would decline with increasing body weight, the numerical increase in requirement shows that the diet composition is an important factor, which affected the threonine requirement observed between dietary treatments. The barley-casein diet consisted of 50% of the dietary threonine from barley and 50% from casein, while for the casein diet all dietary threonine came from casein. As seen in Chapter 4 the ileal digestibility of threonine in the barley-casein diet was ten percent lower than that for the casein-based diet. The difference in threonine digestibility between the two diet ingredients will have an important impact on the threonine requirement calculated from these two diets components.

The results of this experiment showed that based on the indicator amino acid oxidation technique, threonine requirement appeared to be higher for growing pigs receiving a barley-casein diet relative to a casein-based diet. However, the difference was not significant. Two methods, the two-phase linear crossover model (Ball & Bayley 1984) and the quadratic fit model (Parr et al. 2003, Baker et al. 2002) were used to determine threonine requirements for both diets. Because of the limited number of threonine intakes (six) that were used to determine requirement in this experiment, it was important to validate the threonine requirement using several methods. Hence, both the plateau value from the two-phase model and the quadratic fit were applied to the data set. For the quadratic fit model the amino acid requirement was determined as the point of intersection between the quadratic curve and the broken line (Parr et al. 2003, Baker et al. 2002). As seen in Figure 5.3.4, the data from this experiment can be fitted to both the two-phase linear regression model and to the quadratic model. Although both models fit, based on the R-square values it was obvious that the two-phase linear model provided the best fit for the model.

There are various methods used for the determination of amino acid requirement, which all involve feeding graded levels of the test amino acid and looking at changes in particular biological parameter(s). Nitrogen balance and growth are classical methods that have been used for amino acid determination in both human and animals (Rose 1957, Kang-Lee & Harper 1978, Leibholz 1988, Heger et al. 2002). The primary argument against these classical methods is that the measured parameters are indirect measurements and therefore may be insensitive (Zello et al. 1995, Brunton et al. 1998, Pencharz & Ball, 2003). Then there is the direct oxidation approach where the test amino acid was used as the tracer (Stephen & Waterlow 1968, El-Khoury et al. 1994). However, the direct oxidation method is not an effective measurement for all

indispensable amino acids due to complex oxidative pathways for some amino acids. For example, carbons in threonine are not directly oxidized to carbon dioxide; rather they are sequestered by glycine and so threonine requirement is underestimated by direct oxidation method (Ballevre et al. 1991). Thus, for determination of threonine requirement, the indicator amino acid oxidation (IAAO) is more appriopriate than direct oxidation.

The IAAO technique has been successfully utilized for determination of amino acid requirements in pigs and humans (Bertolo et al. 1998, Kim et al. 1983a & b). In most cases the tracer was infused intravenously, and blood samples were collected for measurement of amino acid kinetics. However, a less invasive procedure is to provide the tracer infusion orally rather than intravenously. Studies by Cvitkovic et al. (2000) and Kriengsinyos et al. (2002) in pigs and humans, respectively, have found no difference in the routes (intravenous vs. oral) of isotope infusion on amino acid requirement estimation using the IAAO technique. However, phenylalanine kinetics were affected by the route of isotope infusion, with a lower calculated estimate of phenylalanine oxidation and flux when the tracer was provided intravenously (Kriengsinyos et al. 2002). Splanchnic utilization of phenylalanine was cited as the most likely reason for the observed difference. First-pass splanchnic uptake of phenylalanine ranges from 29 to 58% (Biolo et al. 1992, Stoll et al. 1999). But even with these differences in phenylalanine kinetics, overall there was no difference in the estimate of amino acid requirement via route of infusion. As such, for this experiment we opted to provide the tracer orally rather than intravenously. Further evidences which support the claim that route of infusion does not alter the amino acid requirement can be seen by comparing the work of Kim et al. (1983a) to that of Bertolo et al. (1998). The threonine requirement in orally fed pigs (~2.5 kg) was previously determined by indicator amino acid oxidation using ^{14}C-phenylalanine provided orally (Kim et al. 1983a). The broken-line regression model gave a mean threonine requirement of 6.0 g/kg diet (~0.58 g THR/kg BW). Then later the oral threonine requirement was determined for 2.55 kg pigs, which were fed by intragastric catheters and isotope infusion was provided intravenously (Bertolo et al. 1998). For this experiment, oral threonine requirement was determined to be 0.51 g THR/kg BW.

The IAAO method has been shown to be quite efficient in determining amino acid requirement without the need for blood sampling and further isolation of the radioactive label. Chen (1997) and Law (2000) used measured parameters such as expired CO_2, phenylalanine oxidation, phenylalanine oxidation as a percentage of dose and phenylalanine balance, all of which gave

similar estimates of threonine requirement; but in both studies, phenylalanine oxidation as a percentage of dose was the least variable parameter. Thus, based on the results from those studies, in this study we opted not to collect blood samples because measuring phenylalanine oxidation as a percentage of dose has been shown to provide more reliable data. Further argument is that the collection of CO_2 is the last step in a chain of events: the expiration of CO_2 occurs after oxidation has taken place in the mitochondria, and the $^{14}CO_2$ has traveled through the pig and out of the lungs. Therefore, when the expired CO_2 has reached a plateau, all other measures must have reached a steady state. Additionally, the use of catheters has proven to be problematic in growing pigs, where experience within the research group has shown that despite precautionary measures, growing pigs are apt to pull out their catheters, and there are continuous problems with maintaining patent catheters.

For this experiment, at least two days adaptation to each dietary threonine level was considered sufficient time to obtain reliable measurements of phenylalanine oxidation. In this experiment, it was noted that when increasing or decreasing the threonine content of the diet, phenylalanine oxidation decreased and increased, accordingly, to a new level within two days, and was not significantly different there after. Kim et al. (1983) and Mohn et al. (2001) also observed similar two-day amino acid adaptation responses in growing pigs. Mohn et al. (2001) found that for 30 kg swine given lysine-deficient diets, when the lysine content was reduced from 123 to 56% of the NRC (1998) recommendation, phenylalanine oxidation increased to a new level within two days, after which it did not change significantly ($p>0.1$) when the lower lysine intake was maintained for 3, 4 or 6 days (total of 18 observations). Conversely, when the dietary lysine level was increased again to 123% of NRC (1998), the phenylalanine oxidation dropped to a low level within two days, and stayed constant thereafter for 3, 4 and 6 days (16 observations). This two day adaptation period has also been noted for sows (Mohn et al. 2001) and for adult humans (Thorpe et al. 1999). This short adaptation period for the indicator amino acid oxidation technique allows us to measure several threonine levels within the same animal over a short period of time. Thus, we were able to determine individual pig threonine requirement for casein and barley-casein diets within the same animal. By using repeated measure analysis, testing all dietary treatments within the same animal helps to reduce the experimental variation, thereby increasing the sensitivity of the analysis.

In this experiment we attempted to determine individual pig threonine requirements. The variability of the threonine requirements within diets was quite large, with a CV of 10% to 20%

of the mean. The variability observed in this experiment was similar to that found for the lysine requirement of pigs of similar age (Mohn et al 2001). This is the first known experiment that measures dietary amino acid requirement of two complete diets within the same pig. Determination of individual animal amino acid requirement allows for estimation of variation in requirements within the population. For the barley treatment, we were not able to estimate breakpoints for two of the six pigs because they did not exhibit a plateau in $^{14}CO_2$ in breath for enough THR intakes. The inability to establish plateau in expired $^{14}CO_2$ breath resulted in failure to obtain plateaus in the two-phase regression graphs for two of the six pigs. For the remaining four pigs, breakpoint estimate of threonine was 0.382 (SD 0.064) g THR/kg BW with upper 95% and lower confidence limits of 0.281 g THR/kg BW and 0.483 g THR/kg BW, respectively. The overall mean threonine requirement between barley and casein diet showed 10% higher requirement from barley within repeated measure sensitivity of the method. The average differences (0.027 g THR/kg BW) in threonine requirement between the two diets showed higher threonine requirement for the barley diet compared to the casein diet. The IAAO method can be used to determine individual animal amino acid requirements because it allows multiple measurements to be performed within the same animal in quick succession, without changing the animal's physiological state during the experiment (Ball et al. 2002). As Ball et al. (2002) have stated there are several important advantages to determining individual animal amino acid requirement, such as better feed formulation approaches and assessment of the economic viability of feeding regimens that supply amino acids at above or below the average requirement of a herd of pigs.

5.5 Conclusions

To summarize, the results of this experiment showed that on a total digestibility basis, threonine requirements were greater for growing pigs fed barley relative to casein-based diet. However, when diets were compared on true and ileal threonine basis the threonine requirements were greater for pigs fed casein-based diets. This indicated that the overall greater threonine requirement from the barley-based diet was partly due to the higher endogenous threonine losses. However, the similar decline of oxidation from both diets indicated similar utilization of the free threonine that was added to the diets. In conclusion, when formulating diets for growing pigs both digestibility and anti-nutritive effects of the feed ingredients in the diet need to be considered. As seen in this experiment the addition of free threonine to a diet that is rich in anti-nutritional factor will not impair the utilization of this amino acid. More research is needed in order to evaluate the

change in efficiency of re-utilization of endogenous threonine when feeding feedstuffs that contain high anti-nutritional factors. Based on the current results of this experiment, it is expected that a greater inclusion of barley and or more animals (in this experiment, barley treatment had n = 4, while casein treatment had n = 6) would show statistical differences between threonine requirement from casein and barley diets.

Chapter 6

GENERAL SUMMARY AND DISCUSSION: FUTURE DIRECTIONS

Threonine has been shown to be important for the development and function of the gastrointestinal tract (Bertolo et al. 1998). As was demonstrated in this project, there is a link between anti-nutritional factors (ANF) in the diet and ileal threonine loss and mucin output, where greater mucin output was associated with more ileal threonine loss. We also attempted to show that this ileal threonine loss meant greater a threonine requirement for growing pigs. Although further study is required before any generalized conclusion can be drawn relating ANF, mucin output and amino acid requirements, the main outcomes from this project do suggest a strong relationship between mucus-stimulating ANF and threonine requirement. One of the primary findings of this project was the role of fibre in the diet. Fibre content of the diet did seem to impact on mucin and threonine output. For future study, it would be of interest to determine whether there was a minimal level of fibre required in the diet to affect mucin and threonine output and whether the relationship is linear.

Results from this project clearly indicate that barley does impact mucin output and may affect threonine requirement. One area for future study would be the dose response effect of barley on threonine requirement in pigs and in humans. Barley is a food source not only for pigs but is also consumed by humans. Barley, due to its beta-glucan content, has been shown to be beneficial to humans in reducing plasma cholesterol and moderate the immune system (Hallfrisch et al. 1995, Kalra & Jood 2001). A question that may arise from this experiment is whether barley would affect mucin output in humans. If it was proven that barley could affect mucin output in humans, then the question then becomes what is the balance in achieving the benefits from barley because of its beta-glucan content without reaping too much of a detrimental effect due to its high insoluble fibre content.

The effect of kidney beans on mucin output is another avenue that could be further explored based on the result of this research. The results from this study indicate that kidney beanss increase mucin output and threonine loss. Beans are a high protein food source that is consumed both by humans and animals. The lectins in some beans such as red kidney beanss have been shown to be quite potent in stimulating mucus. For vegetarians, whose primary source of protein is legume, the impact on threonine maybe very important for meeting protein, particularly

threonine needs. However, a minimal level for lectins-induced effects from beans in pigs has not been established. It would be interesting to determine if the minimum threshold level would be similar to those established in murine animals.

Further work is required showing a direct relationship between threonine requirement and mucin output. In future studies that attempt to link threonine requirement and mucin output, researchers should look at a model that allows simultaneous testing of mucin output and threonine requirement. A model such as end-to-end-ileorectal anastomosis, which allows total collection of digesta would probably work better than the simple-T-cannula method. The T-cannula method only allows sample collection of the digesta and therefore requires the use of digestibility marker to determine total flow. One of the primary concerns with the T-cannula is the internal diameter of the cannula. With a high fibre diet, these digesta samples may not be a true homogeneous representative of the digesta (Sauer & de Lange 1992, Nyachoti et al. 1997). However, the ileo-rectal anastomosis technique may not work well for long-term study because of concerns regarding the physiological and nutritional impact of this surgery on the animal. Thus, first there is the need for a digesta collection model that allows for proper representation of digesta regardless of the diet. The digesta (for mucin) collection method should then be combined with simultaneous IAAO method. One question about this combination would be adaptation period. For the IAAO method it has been demonstrated on various occasions, that two days adaptation between dietary levels is adequate to elicit a measurable response in the test animal (Kim et al. 1983, Mohn et al. 2001). However, for mucin output there are no references indicating the time required for change in mucosal secretion with change in diet; is known that enterocytes require three to four days for turnover (Patience et al. 1995, Croft et al. 1976). Therefore, it would also be interesting to see if there was a measurable correlation between days of adaptation to the diet as measured by IAAO and mucosal secretion as measured by cannulation technique.

As stated by various researchers, it is important to be able to determine amino acid digestibility and availability in the pig, not only for the purposes of efficient dietary formulation for this species but also to assess protein quality in foods for humans (Rowan et al. 1994, Moughan 2003). Thus, the result of this current project has implications not only for the growing pig but also for our understanding of diet and gastrointestinal function in humans.

Dietary supplementation of total enteral nutrition formulas is proposed to have many benefits, including the maintenance of small intestinal and large bowel function (Silk 1987, Scheppach et

al. 1990). However, nutrient losses from the small intestine have been reported to increase with the ingestion of dietary fibre (Steinhart 1992, Zhang et al. 1992), which may be detrimental, especially in critically ill patients who may not be meeting their nutrient requirements (McBurney 1994). Adult humans with ileostomies are used to study digestibility of dietary amino acids as determined at the terminal ileum or over the entire digestive tract (Rowan et al. 1994, Lien 1995). But there are limitations with the ileostamate for determining ileal digestibility values due to the microbial colonization that occurs following surgery. Therefore, the neonatal pig and growing pig are important animal models for the study of protein digestion in man.

Fibre is known to have many health benefits. For example, high fibre diets have been used in the treatment and prevention of some gastrointestinal disorders such as inflammatory bowel disease and diverticular disease. In chronic feeding studies the use of insoluble fibre preparations such as wheat bran and cellulose fibres have been shown to slow glucose and cholesterol absorption through alteration in mucin quantity (Satchithanandam et al. 1990). However, too much fibre can also lead to exacerbated health risks for certain gastrointestinal disorders; thus proper balance in fibre intake is needed for treatment and prevention of gastrointestinal disorders. For adult humans in North America, the recommended daily- fibre intake is 25-35g per day. This recommendation is probably appropriate for the general population who also consume a high quality protein diet; however, for those subsets of people who are vegetarians they may be at risk for health problems. Vegetarians usually consume protein sources that are of lower quality than non-vegetarians. Their diets are also more fibrous diets such that their daily fibre intakes are normally greater than the recommended intake. Based on the finding of our research, these individuals may be at risk for amino acid deficiencies. Because of the integral role threonine plays in the structure and functioning of the gastrointestinal tract, deficiency in threonine may result in the gastrointestinal tract being more susceptible to pathogenic invasion, and digestive proteases. This consequence, would also affect the digestion and absorption of nutrients. Another subset of people who may be at risk are individuals with certain gastrointestinal disorders, for these people, some fibre in the diet is also beneficial, but proper care has to be taken to ensure that there are more benefit than harm due to the presence of fibre in their diet.

In conclusion, the results of this project have shown that; of common anti-nutritional factors in growing pig's diets, fibre content caused the greatest mucin output and contributed to the highest losses of threonine. It was also shown that threonine requirement was higher for growing pigs fed a barley-based diet, which was high in fibre, relative to a casein-based diet.

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